Abstract

Survivin, a family member of the inhibitor of apoptosis proteins that is expressed during mitosis in a cell cycle–dependent manner and localized to different components of the mitotic apparatus, plays an important role in both cell division and inhibition of apoptosis. Survivin is expressed in a vast majority of human cancers, but not in normal adult tissues. Survivin expression is often correlated with poor prognosis in a wide variety of cancer patients. These features make survivin an attractive target against which cancer therapeutics could be developed. We have identified a survivin antisense oligonucleotide (ASO) that potently downregulated survivin expression in human cancer cells derived from lung, colon, pancreas, liver, breast, prostate, ovary, cervix, skin, and brain as measured by quantitative RT-PCR and immunoblotting analysis. Specific inhibition of survivin expression in multiple cancer cell lines by this ASO (LY2181308) induced caspase-3–dependent apoptosis, cell cycle arrest in the G2-M phase, and multinucleated cells. We also showed that inhibition of survivin expression by LY2181308 sensitized tumor cells to chemotherapeutic-induced apoptosis. Most importantly, in an in vivo human xenograft tumor model, LY2181308 produced significant antitumor activity as compared with saline or its sequence-specific control oligonucleotide and sensitized to gemcitabine, paclitaxel, and docetaxel. Furthermore, we showed that this antitumor activity was associated with significant inhibition of survivin expression in these xenograft tumors. On the basis of these, LY2181308 is being evaluated in a clinical setting (Phase II) in combination with docetaxel for the treatment of prostate cancer.

Mol Cancer Ther; 10(2); 221–32. ©2011 AACR.

Introduction

Apoptosis, or programmed cell death, is a highly organized physiologic event that plays an essential role in controlling cell number in many normal processes, ranging from fetal development to adult tissue homeostasis (1, 2). Abnormal regulation of apoptosis has been implicated in the onset of wide range of diseases including cancer. The process of apoptosis is tightly regulated by a number of gene products that promote or block cell death at different stages of apoptosis. Approaches to resensitize cancer cells to apoptosis represent an important future strategy for cancer treatment (2), which include restoring lost apoptotic intermediates, inactivating antiapoptotic proteins, triggering apoptosis pathways that remain intact in cancer cells, and inducing apoptosis by targeting specific tumorigenic lesions (2, 3).

Members of the inhibitor of apoptosis proteins (IAP) family are evolutionary conserved and play an important role in the negative regulation of apoptosis (4). Survivin, the smallest member of the IAP family of proteins with a single BIR domain and carboxyl terminal α-helix, is perhaps one of the most prominent proteins associated with a wide variety of cancers and has attracted significant attention in recent years (5–9). Survivin expression is cell cycle regulated with peak expression in the G2-M phase and it localizes to various components of the mitotic apparatus (9, 10). Immunofluorescence and confocal microscopy techniques showed that survivin localizes to kinetochores and centrosomes (microtubule-organizing centers) during prophase, spindle microtubules during metaphase, central spindle midzone during anaphase, and midbodies during late telophase (11). One of the most outstanding features of survivin is its abnormal overexpression in a vast majority of cancers, but not in normal, terminally differentiated tissues. Survivin is strongly and broadly expressed in embryonic and fetal tissues; however, it is undetectable in most terminally differentiated tissues except in thymus, testis, angiogenic endothelium, and intestinal crypt cells (5, 7–9, 12–15).
Survivin plays key role in both negative regulation of apoptosis for extending the lifespan of neoplastic cells and the mechanism of cell division. Several lines of experimental evidence have elegantly shown the role of survivin in the regulation of apoptosis (4, 16–20). However, the precise mechanism by which survivin inhibits apoptosis is not very clear. Retrospective analysis of clinical samples has provided strong evidence that survivin expression predicts a reduced apoptotic index in tumors and poor patient survival (5, 21–30). Because of its differential expression in tumors versus normal tissues and its role in apoptosis for maintaining cell viability, survivin represents a promising drug target for cancer therapy. Several in vitro and in vivo studies showed a T-cell-mediated cytolytic response against survivin peptides (31). Furthermore, the presence of HLA class I–restricted cytolytic T cells against survivin peptides have been detected in breast cancer, melanoma, and leukemia patients (32, 33). Thus, an immunotherapy approach using survivin peptide-specific cytolytic T cells may offer therapeutic benefits (34). On the contrary, survivin crystal structure has been resolved and has been shown to exist as a homodimer in the cytoplasm (35–37). Historically, the identification of small molecule inhibitors of cytoplasmic protein–protein interaction has been challenging. As a result, therapeutic targeting of proteins, such as survivin, by conventional approach remained to be very difficult. Novel approaches such as antisense and siRNA offer attractive alternatives to regulate gene expression. Therefore, it is hypothesized that blocking the expression of survivin with a molecule such as an antisense oligonucleotide (ASO) will restore default cell-death checkpoints and will eliminate cancer cells. Here we report that the ASO, LY2181308, potently inhibited survivin mRNA and protein expression in various tumor cell lines. Inhibition of survivin expression in tumor cell lines by this ASO resulted in the induction of caspase-3 activity, cell cycle arrest, and failure of cell division. Importantly, LY2181308 significantly inhibited expression of survivin protein in multiple human xenograft tumors and this resulted in significant inhibition of human xenograft tumor growth when administered intravenously and sensitized tumors to gemcitabine, paclitaxel, and docetaxel.

Materials and Methods

Cell lines and reagents

Human cervical carcinoma HeLa; bladder carcinoma T24; colon carcinoma HCT-116; non–small cell lung cancer A549; prostate carcinoma PC3; breast MDA MB231; ovarian carcinoma OVCAR, SKOV3, and IGROV1; glioblastoma U-87 MG; osteosarcoma U2OS; lung NCI-H460 (American Type Culture Collection); and HUVEC cells (Clonetics) were maintained in culture according to the suppliers recommendations. Human melanoma YUSAC-2 cells were obtained from Dr. Ruth Halaban (Yale University, Department of Dermatology, New Haven, CT; ref. 38) and were cultured in Ham’s F12 media containing 7% FBS and 2 mmol/L l-glutamine. No independent authentication of these cell lines were done by the authors. LY2181308 (5′-TGTGCTATATCTGTAAT-3′) and mismatch control (MM control; 5′-TAAGCTGGTCTATGTGT-3′) oligonucleotides were synthesized and were dissolved in phosphate buffered saline (PBS) or normal saline. The underlined sequence represents 2′-O-methoxymethyl (2′-MOE) modifications on a uniform phosphorothioate backbone oligonucleotide. Cytosines at position 5 and 10 are 5-methyl substituted. A BLAST search of public databases of human genes was performed to exclude homology of the antisense and MM control oligonucleotides to other genes.

Transfection of tumor cell lines

Cells were grown at a 65% to 75% density overnight in a 96-well plate or 6-well plate or 10-cm tissue culture dishes. Transfection of tumor cell lines with LY2181308, survivin ASO and MM control oligonucleotide was performed using lipofectin reagent (Invitrogen) and serum-free OPTIMEM (Gibco Life Technologies/Invitrogen). Briefly, 3 μL of lipofectin were used for each 100 nmol/L ASO. Lipofectin reagent and OPTIMEM were preincubated for 30 minutes at room temperature. ASOs were then added to mixture and incubated for 15 minutes following 2-fold dilutions of initial mixture using plain OPTIMEM (no lipofectin added). Initial concentration for ASO was 400 nmol/L and 7 additional concentrations were obtained after dilutions. A mixture of lipofectin/OPTIMEM was used for untreated controls. Transfection mixture was then removed following 4 to 6 hours of incubation at 37°C and replaced with complete medium. Cells were incubated in complete medium for 24 to 72 hours prior to harvest. Following 24 hours of incubation with oligonucleotide, tumor cells were processed for either total RNA or total protein isolation. Survivin mRNA levels were measured by quantitative RT-PCR using a survivin-specific PCR primer/probe set. Survivin protein levels were analyzed by immunoblotting with a survivin-specific antibody.

Real-time RT-PCR

Total RNA was isolated from cells by using the RNeasy 96 Kit (QIAGEN) according to the manufacturer’s instructions. RNA was then diluted 1:2 or 1:10 with RNase and DNase-free water. For cDNA synthesis and RT-PCR amplification, Taqman One-Step RT-PCR Master Mix Reagents Kit and diluted RNA were used in the ABI Prism 7700 or 7900 Sequence Detection System (Applied Biosystems). Amplification of survivin cDNA was performed using the following Taqman probe primers (synthesized by Biosearch Technologies): forward primer p1115′-GCACACTTCCAGGGTTATTCC-3′; reverse primer p1125′-TCTCCCTTCTCAAGACATTTGCTAAGG-3′; and the Taqman probe 5′-6-FAM d(TGTGTCGCCACAGCCTTCTGTO)BHQQ-1′. Relative gene expression
was quantized as described in User Bulletin 2 using ABI Prism 7700 Sequence Detection System (Applied Biosystems). Amplification of GAPDH cDNA was done separately by using Taqman GAPDH Control Reagents (Applied Biosystems) and diluted RNA as indicated before. Survivin/GAPDH mRNA ratios in compound-treated samples were compared with mock-transfected control and expressed as a percentage inhibition. Data were analyzed by a nonlinear regression analysis using GraphPad Prism software to calculate inhibitory concentrations of 50% (IC50).

Immunoblotting

For confirming effects of compound treatments on protein levels, cells were harvested by washing twice with 1× PBS and then by lysing in 100 μL RIPA buffer [20 mmol/L Tris-HCl (pH 7.4), 5 mmol/L EDTA, 50 mmol/L NaCl, 10 mmol/L sodium pyrophosphate, 50 mmol/L NaF, and 1% NP40] with 2 mmol/L Na2VO4 and 1 tablet Complete Mini protease inhibitor cocktail (Roche), and protease inhibitor cocktail II (Sigma) freshly added. Xenograft tumors excised from nude mice were homogenized in RIPA buffer using Bio101 tissue homogenizer beads and FastTrak instrument (MP Biomedical). Cell lysates were cleared by centrifugation at 14,000 rpm for 30 minutes at 4°C. BCA protein assay (Pierce/ThermoFisher) was done to determine protein concentration. Between 30 and 60 μg of total protein were resolved by SDS 10% to 20% gradient Tris-Glycine gel electrophoresis (Novex/Invitrogen) and transferred to Immobilon PVDF membrane (Millipore) by semidry electrophoretic blotting transfer buffer system (Owl/ThermoFisher). Membranes were blocked with Tris-buffered saline with 0.1% Tween-20 (TBST) and 5% nonfat milk. Proteins were visualized by incubation with survivin primary antibodies at a dilution of 1 mg/mL (R&D Systems) and/or actin primary antibodies 1:10,000 (Sigma) overnight at 4°C, followed by incubation with horseradish peroxidase conjugated secondary antibody at a 1:1,000 dilution in TBST for 2 hours at room temperature. Membranes were then washed 3 times with TBST and resolved using Supersignal West Pico Chemiluminescence detection system (Pierce/ThermoFisher). Secondary antibodies used were donkey anti-rabbit horseradish peroxidase conjugated for survivin detection (Amersham/GE Life-sciences) and goat anti-mouse horseradish peroxidase conjugated for actin detection (BioRad). Other primary antibodies used included anti-XIAP and anti–Bcl-xL, both used at a 1:100 dilution (Cell Signaling), and anti-Bcl-2 at a dilution of 1:500 (Santa Cruz Biotechnologies). Fluorescent images from immunoblotting were captured directly via a cooled charged coupled device camera-equipped BioRad’s Fluor-S instrument. Quantization of survivin protein was carried out using Quantity One software (BioRad). Survivin protein levels for the LY2181308-treated samples were compared with mock-transfected control or MM control–treated tumor samples and expressed as a percentage inhibition. Data were analyzed by a nonlinear regression analysis using GraphPad Prism software to calculate IC50s.

Determination of caspase-3 activity and apoptosis

Whole-cell caspase-3 activity assays were carried out as described previously (39). Briefly, 3× lysis/reaction buffer containing 150 mmol/L HEPES, 1.5% NP40, 450 mmol/L NaCl, 150 mmol/L KCl, 30 mmol/L MgCl2, 1.2 mmol/L EGTA, 30% sucrose, 0.3% CHAPS with freshly added phenylmethylsulfonylfluoride, DTT, and DEVD-AMC (Biomol GMBH) was added to the wells. Following 1-hour incubation at 37°C in 5% CO2 the enzymatic activity was measured by reading the plates in a CytoFluor multiwell plate reader at excitation 360 nm and emission 460 nm (gain of 50). Data were expressed as a percent increase in caspase-3 activity for compound-treated samples when compared with mock-transfected control values. Apoptosis assay was carried out using Cell Death Detection ELISA kit (Roche) according to the manufacturer’s instructions. Enrichment factor was calculated by dividing the mean reading of compound-treated samples by the mean reading of mock-transfected control sample. Enrichment factor of more than 2 is an indicator of cells undergoing apoptosis.

Flow cytometry

For cell cycle analysis, cells were harvested by pooling floating cells in the media and attached cells, washed with ice cold PBS, and fixed with 70% chilled ethanol by overnight incubation at −20°C. The cells were resuspended in 100 μL of PBS containing 5 mg/mL RNase-A (DNase-1 free), followed by the addition of 900 μL of 50 μg/mL propidium iodide (Sigma). The cells were incubated on ice for 30 minutes in the dark and then filtered through a 35-μm cell strainer cap before analyzing for the DNA content using Becton Dickinson ExCalibur Flow Cytometer (Becton Dickinson). ModFit LT software was used to estimate the fraction of each cell population in the G1, S, and G2-M phases of the cell cycle.

Determination of nuclear morphology

To evaluate effect of compound treatments on nuclear morphology, HeLa cells were transfected with LY2181308 and MM control oligonucleotide for 48 hours, cells were fixed with 10% phosphate buffer formalin on 8 chamber slides (Nalge Nunc). After washing with PBS, cells were incubated with 5 μg/mL Hoechst 33342 (Sigma) for 15 minutes. Cells were mounted and imaged with inverted Nikon microscope.

Xenograft studies

For in vivo studies human glioblastoma U-87 MG (40), and human melanoma YUSAC-2 (20, 38) xenograft tumor models were used. A total of 10 CD1 nu/nu (Charles River) mice were used for each group. The Eli Lilly and Company Animal Care and Use Committee approved all the experimental protocols. Just before
implantation, animals were irradiated (450 total body irradiation) and cells were mixed in matrigel (1:1). A total of $6 \times 10^6$ (U-87 MG), $4 \times 10^6$ (YUSAC-2), and $5 \times 10^6$ (NCI-H460) tumor cells in a 0.2 mL volume were injected subcutaneously in the left rear flank. Treatment with LY2181308 or MM control oligonucleotide dissolved in vehicle or vehicle alone (0.9% NaCl injection grade) via intraperitoneal or intravenous injection was initiated when tumor volume reached to $100 \pm 50$ mm$^3$. When animals were sacrificed, tumors were removed and flash frozen for determination of survivin protein levels by immunoblotting or fixed in 3% paraformaldehyde in PBS, embedded in paraffin, and subjected to haematoxylin and eosin (H&E) or immunohistochemical staining using cyclin B1 (Dako) antibody. For a time-dependent survivin inhibition study, tumors were collected 4, 8, 24, 48, 72, and 96 hours ($n = 8$ per group) after dosing, homogenized in RIPA buffer and subjected to immunoblotting using human survivin and XIAP-specific antibodies. For a single agent efficacy studies, all animals received a single loading dose of 50 mg/kg of LY2181308 or MM control oligonucleotide followed by 25 mg/kg every other day for 3 weeks. Every other day dosing schedule was initiated 1 day after the loading dose of 50 mg/kg. For combination studies, treatment with suboptimal doses of gemcitabine or docetaxel was initiated on the day after the loading dose. Gemicatbine was administered intraperitoneally at 2.5 mg/kg every third day for a total of 4 doses. Paclitaxel was administered intravenously at 5 mg/kg every fifth day for a total of 4 doses. Docetaxel was administered intravenously at 5 mg/kg every fourth day for a total of 3 doses. Bidimensional measurements were done twice a week and tumor volumes were calculated on the basis of the following formula: Tumor Volume = $(L \times W^2 \times \pi/6)$ where $L$ is mid-axis length and $W$ is mid-axis width. Tumor volume data were transformed to a log scale to equalize variance across time and treatment groups. The data were analyzed using a 2-way repeated measures analysis of variance (ANOVA) by time and treatment using SAS PROC MIXED software (SAS Institute). The correlation model for the repeated measures was AR1 (autoregressive of order 1). Treatment groups were compared at each time point. The data were plotted as means and SE for each treatment group versus time. The presence of synergy in in vivo combination therapies was assessed on the tumor growth delay (TGD) scale. The time to reach a specified tumor size (1,500, 2,000, or 2,500 mg in this study) was determined for each animal. Tumors that did not reach that size were included in the analysis as right-censored values. Maximum likelihood analysis assuming a Weibull distribution was used to calculate mean times and SE for each treatment group. Tumor growth delay was defined as the difference in mean times between each of the treated groups and the control group. A combination therapy was determined to have a synergistic effect if its TGD was significantly more than the sum of the TGDs for the individual therapies.

Results

**LY2181308 specifically downregulates survivin mRNA and protein expression**

Two screens consisting of determining the amount of survivin mRNA inhibition caused by the transfection of 117 different 2'-MOE chimeric phosphorothiate oligonucleotides were done in T24 human bladder carcinoma tumor cells to identify effective ASOs against survivin by quantitative real-time RT-PCR. Details of screening procedure and results are described elsewhere (41). Briefly, oligonucleotides with greater than 50% inhibition of survivin mRNA were confirmed for their activity by a subsequent round of RT-PCR. These molecules were further followed up by a dose–response analysis and confirmation by Northern blot hybridization to identify most potent antisense molecule, LY2181308. A MM control oligonucleotide for LY2181308 was prepared by incorporating six MM bases while maintaining the same G/C content as the antisense molecule.

Survivin is expressed in a wide variety of tumor cell lines (5). To evaluate how broadly LY2181308 is effective, multiple tumor cell lines were analyzed for the inhibition of survivin expression by LY2181308. Human tumor cells were grown as a monolayer and transfected with the LY2181308 and MM control oligonucleotide. LY2181308 potently inhibited the expression of survivin mRNA (Supplementary Fig. S1) and protein with IC$_{50S}$ ranging from 0.008 to 0.1 μmol/L (Table 1) in a wide variety of tumor cell lines, whereas MM control oligonucleotide had IC$_{50S}$ greater than 0.4 μmol/L in all cell lines evaluated. Furthermore, LY2181308 in a sequence-specific and concentration-dependent manner inhibited the expression of survivin protein and not of other antiapoptotic proteins such as XIAP, Bcl-2, and Bcl-xL in HeLa cells (Fig. 1).

**Effect of survivin inhibition by LY2181308 on cell cycle progression and apoptosis**

Survivin is important for regulation of the cell cycle. Survivin expression is cell cycle regulated with the highest expression observed during the G$_2$-M phase of the cell cycle (42, 43). Therefore, inhibition of survivin expression would be expected to cause G$_2$-M arrest. As shown in Figure 2a, LY2181308 treatment arrested HeLa cells in the G$_2$-M phase of the cell cycle in a concentration-dependent manner. In contrast, MM control had little or no effect on DNA content.

Survivin also plays a key role in the regulation of cytokinesis. Thus, it is expected that the inhibition of survivin would result in the failure of cytokinesis and give rise to cells with multiple nuclei (polyploid cells). HeLa cells treated with LY2181308 and MM control oligonucleotide for 48 hours, stained with Hoechst 33342 dye. As shown in Figure 2b, transfection of HeLa
cells with the LY2181308 results in a concentration-dependent increase in multinucleated cells (Fig. 2B, ii). Morphologically, these cells became abnormally large and flattened and accumulated multiple nuclei as analyzed by fluorescence microscopy of Hoechst 33342 stained cells, whereas HeLa cells transfected with control MM control had normal nuclear morphology (Fig. 2B, i). This observation is consistent with a critical role of survivin in mitosis exemplified by the phenotype of knockout mice (44). Homozygous knockout of the survivin gene results in early embryonic lethality due to catastrophic defect of microtubule assembly, the absence of mitotic spindles, and the formation of multinucleated cells during development (44).

To evaluate the effects of survivin inhibition on cell death, cultured tumor cell lines were transfected with LY2181308 or MM control oligonucleotide and caspase-3 activity (39) was measured at various times thereafter. Multiple tumor cell lines were evaluated in this assay and were sensitive to LY2181308-induced caspase-3 activity. Figure 2c shows representative data at 48 hours using HeLa cells. LY2181308 reproducibly induced caspase-3 activity in a sequence-specific and concentration-dependent manner in HeLa cells. This increased caspase-3 activity resulted in apoptotic cell death in HeLa cells as measured by detection of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates using Cell Death Detection ELISA $^+$ PLUS kit (Supplementary Fig. S2). Evaluation of sub-G1 peak by flow cytometry analysis also revealed LY2181308-mediated cell death (data not shown). In contrast, MM control did not significantly alter the sub-G1 population.

**Effect of LY2181308 on pharmacodynamic parameters in xenograft tumors**

To investigate the effect of LY2181308 on survivin inhibition in xenograft tumors, we used human glioblastoma (U-87 MG) and human melanoma (YUSAC-2) xenograft models. To understand kinetics of survivin inhibition, a time-course study was done in U-87 MG xenograft tumors following a single administration of LY2181308 or MM control oligonucleotide at the rate of 50 mg/kg. As survivin is primarily expressed in tumors but not in normal tissues and LY2181308 does not inhibit XIAP protein expression, we normalized survivin protein levels to XIAP. Normalization of survivin proteins to XIAP using antibody specific to human XIAP instead of antibodies to actin or GAPDH avoided potential artifacts due to proteins contributing from host stroma. Survivin/XIAP ratios in LY2181308 and MM control

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>n</th>
<th>Mean IC50 (nmol/L)</th>
<th>SE</th>
<th>Mean IC50 (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>5</td>
<td>8 3</td>
<td></td>
<td>&gt;400</td>
</tr>
<tr>
<td>T24</td>
<td>3</td>
<td>29 10</td>
<td></td>
<td>&gt;400</td>
</tr>
<tr>
<td>HCT116</td>
<td>3</td>
<td>64 18</td>
<td></td>
<td>&gt;400</td>
</tr>
<tr>
<td>A549</td>
<td>3</td>
<td>49 16</td>
<td></td>
<td>&gt;400</td>
</tr>
<tr>
<td>PC3</td>
<td>3</td>
<td>21 7</td>
<td></td>
<td>&gt;400</td>
</tr>
<tr>
<td>MDA-MB231</td>
<td>2</td>
<td>76 91</td>
<td></td>
<td>&gt;400</td>
</tr>
<tr>
<td>YUSAC-2</td>
<td>3</td>
<td>112 30</td>
<td></td>
<td>&gt;400</td>
</tr>
<tr>
<td>SKOV3</td>
<td>1</td>
<td>11 N/A</td>
<td></td>
<td>&gt;400</td>
</tr>
<tr>
<td>OVCAR</td>
<td>1</td>
<td>36 N/A</td>
<td></td>
<td>&gt;400</td>
</tr>
<tr>
<td>IGROV</td>
<td>1</td>
<td>41 16</td>
<td></td>
<td>&gt;400</td>
</tr>
<tr>
<td>U-87 MG</td>
<td>1</td>
<td>23 N/A</td>
<td></td>
<td>&gt;400</td>
</tr>
<tr>
<td>HUVEC</td>
<td>1</td>
<td>21 N/A</td>
<td></td>
<td>&gt;400</td>
</tr>
</tbody>
</table>

*Number of experiments.

NOTE: Table shows IC50 for LY2181308 and MM control in their ability to inhibit survivin protein expression in various cell lines. Cells were treated with 8 different concentrations of LY2181308 or MM control oligonucleotide starting at 400 nm. After 24 hours treatment, total protein was isolated and processed for quantitative immunoblotting to detect survivin and actin levels. Percentage inhibition was calculated against the lipofectin control. Data were analyzed by a nonlinear regression analysis using GraphPad Prism software to calculate IC50.

Abbreviation: N/A, not applicable.
tumors were compared and expressed as a percentage of inhibition. As shown in Fig. 3A, significant survivin inhibition was observed by 24 hours and peaked between 24 and 48 hours after a single dose of LY2181308. Inhibition of survivin by LY2181308 at 24 and 48 hours was statistically significant; \( P = 0.013 \) and \( P = 0.007 \), respectively, as determined by one-way ANOVA of the data using JMP software. Statistically significant inhibition of survivin expression by LY2181308 following a single (50 mg/kg) intravenous administration was evident in YUSAC-2 xenograft model (Fig. 3B), and was repeated in U-87 MG xenograft model.

Next, we investigated whether longer-term treatment with LY2181308 would also inhibit survivin expression in the melanoma YUSAC-2 xenograft tumor. In this study, LY2181308 or MM control oligonucleotides were administered intraperitoneally with a loading dose of 50 mg/kg intravenous administration was evident in YUSAC-2 xenograft model (Fig. 3B), and was repeated in U-87 MG xenograft model.

Given survivin’s role in cytokinesis and previously shown ability of LY2181308 to induce polyploidy in HeLa cells in vitro (Fig. 2B), evidence for a similar phenotype induced by LY2181308 in xenografts was sought. Human U-87 MG glioblastoma xenograft tumor-bearing animals were given two doses (50 mg/kg loading dose followed by 25 mg/kg with 1 day holiday in-between) of LY2181308 or MM control oligonucleotides, or saline. Twenty-four hours after the second dose, tumors were stained with H&E and evaluated for the presence of multinucleated cells. Tumors from animals treated with MM control oligonucleotide (Fig. 4A) had morphology similar to the saline-treatment group. However, a significant number of cells in the tumors from LY2181308-treated mice were enlarged and many cells had multiple nuclei (Fig. 4B), suggesting failure of cytokinesis in tumor cells in vivo as a result of survivin inhibition.

Cyclin B1 is a cell cycle regulated protein and is upregulated in the G2-M phase of the cell cycle. Inhibition of survivin expression should produce a G2-M arrest, and thus elevated cyclin B1 expression. Animals bearing YUSAC-2 xenograft tumors were dosed intraperitoneally with a 50-mg/kg loading dose followed by 25 mg/kg every other day for a total of 1 week. Tumors were excised and stained using cyclin B1–specific antibody. Tumor samples treated with LY2181308 had numerous cells with increased cyclin B1 (Fig. 4D and F) staining as compared with samples treated with MM control oligonucleotide.
Intense staining of cyclin B1 in LY2181308-treated samples suggests that cells have arrested in the G2-M phase of the cell cycle as a result of survivin inhibition.

The in vivo antitumor activity of LY2181308 was evaluated in U-87 MG human glioblastoma and YUSAC-2 human melanoma xenograft tumor models. As shown in Fig. 5, administration of MM control did not inhibit YUSAC-2 (Fig. 5A) and U-87 MG (Fig. 5B and C) xenograft tumor growth when compared with vehicle-saline–treated group. In contrast, LY2181308 inhibited tumor growth significantly when administered intravenously in animals bearing YUSAC-2 (Fig. 5A) and U-87 MG (Fig. 5B) xenograft tumors. Moreover, LY2181308 conferred antitumor activity in a dose-dependent manner. U-87 MG xenograft-bearing mice were treated with three different dose levels (2, 10, and 25 mg/kg every other day) of intravenous injection of LY2181308 after a single loading dose of 50 mg/kg. TGD is defined as difference in the time taken for treated versus control tumors to reach 1,000 mm³. A clear dose–response relationship was observed with tumor growth delays of 1.7 ± 1, 4.5 ± 1, and 13.4 ± 0.9 days (mean ± SE) following treatment with LY2181308 doses of 2, 10, and 25 mg/kg, respectively (Fig. 5C), compared with the saline-treatment group. These data clearly show significant, dose-dependent, antitumor activity of the LY2181308.

Antitumor activity of LY2181308 in combination with chemotherapeutic agents

Retrospective analysis of clinical samples has provided strong evidence that survivin expression predicts a reduced apoptotic index in tumors and poor patient survival (5, 7, 45). Survivin overexpression in tumors may contribute to drug resistance by blocking apoptosis (46, 47). Therefore, treatment strategies that target survivin may prove to be effective in overcoming such resistance. To investigate whether downregulation of survivin expression sensitizes HeLa cells to chemotherapy, combination treatment with the survivin ASO, LY2181308, and either gemcitabine or paclitaxel was evaluated. HeLa cells were transfected with LY2181308 or MM control oligonucleotide and treated with either 10 nmol/L gemcitabine or 1.25 nmol/L of paclitaxel for an additional 24 hours. At the end of the incubation period, caspase-3

Figure 3. LY2181308 inhibits survivin expression in human xenograft tumor model. Tumors were harvested at various time points after a single 50 mg/kg (A) or 50 mg/kg followed by 25 mg/kg every other day (B–D) administration of LY2181308 or MM control oligonucleotide to tumor bearing nude mice. Tumors were processed for tumor lysate, followed by quantitative immunoblotting to detect survivin and XIAP levels. XIAP-normalized survivin levels for LY2181308-treated samples were compared with MM control–treated samples and expressed as percentage inhibition. Statistical significance was determined by 1-way ANOVA using JMP software. Inhibition of survivin expression in U-87 MG in a time-dependent manner (A) and YUSAC-2 (B) xenograft models by LY2181308. Representative image of an immunoblot of YUSAC-2 tumor lysate (B, i) and quantization plot (B, ii). Inhibition of survivin expression (D) in YUSAC-2 tumors harvested 24 hours after last (seventh) dose from nude mice treated every other day with 25 mg/kg of LY2181308 is associated with antitumor activity (C).
activity was measured. As shown in Fig. 6A, tumor cells treated with LY2181308 in combination with either gemcitabine or paclitaxel produced greater than additive caspase-3 enzymatic activity versus either agent alone, as determined by the interaction effect in a 2-way ANOVA. In contrast, MM control oligonucleotide treatment did not sensitize tumor cells to either gemcitabine or paclitaxel.

LY2181308 also sensitizes U-87 MG xenograft tumors to gemcitabine or paclitaxel and the lung cancer NCI-H460 xenograft tumors to docetaxel (Fig. 6B). LY2181308 showed statistically significant ($P < 0.01$) enhanced antitumor activity in combination with gemcitabine compared with either agent alone (Fig. 6B). The combination of LY2181308 with gemcitabine provided an additional 3 to 4.6 days of TGD, using thresholds of 1,500, 2,000, or 2,500 mm$^3$, compared with the additive effect (i.e., the sum of the single-agent TGDs). This synergistic effect was statistically significant at all 3 thresholds (48). LY2181308 also showed statistically significant ($P < 0.01$) enhanced antitumor activity in combination with paclitaxel compared with either agent alone (Fig. 6C). The combination of LY2181308 with paclitaxel provided an additional 2.2 to 4.7 days of TGD compared with the additive effect. Statistically significant synergy in the TGD was observed at 1,500 mm$^3$. The combination of LY2181308 with docetaxel was studied in the NCI-H460 xenograft model. Because the study was terminated on day 32, a TGD analysis was not practical. Instead, the data were analyzed by repeated measures ANOVA for tumor growth inhibition. The combination was statistically significantly better than either single agent on days 22 to 32 ($P < 0.017$; Fig. 6D).

Discussion

IAPs, particularly survivin, are highly expressed in most malignancies and play key roles in the survival of neoplastic cells by negatively regulating apoptosis (5). Several studies show that high survivin expression in tumors is associated with lower apoptosis index, increased resistance to chemotherapeutic agents, and poor patient survival. Thus, survivin is an attractive target for cancer therapy. In cells, survivin protein exists as a homodimer and has been proposed to exert its functions primarily through protein–protein interaction (35). Historically, development of small molecule inhibitors targeting intracellular protein–protein interaction has remained challenging and in large part unsuccessful for the development of drugs. We took an approach to inhibit survivin expression using ASOs to develop a therapeutic drug. Here we show that inhibition of
survivin expression or function by LY2181308 induces apoptosis in cancer cell lines and inhibits growth of xenograft tumors in nude mice. Furthermore, we show that LY2181308 sensitizes tumors to chemotherapeutic agents such as gemcitabine, paclitaxel, and docetaxel.

We made several oligonucleotides against human survivin mRNA using a second-generation antisense chemistry designed to inhibit the production of human survivin protein and thus inhibit its functions. First-generation ASOs contain a phosphorothioate (a sulfur substitution of a nonbridging O) backbone. Antisense molecules, based on first-generation chemistry, against survivin are generally weak in their cellular activity and have been used primarily for *in vitro* research (15, 19). Second-generation ASOs contain the phosphorothioate backbone plus the addition of 2'-MOE modification of the ribose of the first 4 and last 4 nucleotides of the ASO (49). These modifications are believed to increase potency due to enhanced affinity for target RNA, increased plasma stability, and decreased toxicity. Inside cells, ASOs bind to selected target mRNA molecules by Watson–Crick base pairing, which results in the destruction of the mRNA by RNase H or the inhibition of mRNA processing or translation. Inhibition of gene expression by these second-generation chimeric ASOs is mainly accomplished by destruction of the target mRNA by recruitment of endogenous RNase H (50). The survivin ASO, LY2181308, is comprised of second-generation chemistry that potently inhibited expression of survivin in all the tumor cell lines tested.

Survivin expression is regulated in a cell cycle manner with a significant increase in expression occurring during the G2-M phase of cell cycle. Thus, survivin overexpression may allow tumor cells to overcome...
the G2-M checkpoint to enforce progression through mitosis. Consistent with survivin function and previous observation, inhibition of survivin expression using LY2181308 arrested tumor cells in the G2-M phase of the cell cycle. Survivin has been identified as one of the chromosomal passenger proteins, which also include the aurora-B kinase, the inner centromere protein and the telophase disk antigen (TD-60). These proteins appear to function as part of a multiprotein complex, which plays multiple roles during cell division. Here we show that inhibition of survivin expression by LY2181308 results in accumulation of multinucleated cells indicating that survivin plays a critical role in cytokinesis. Survivin has also been proposed to be an IAP that may protect tumor cells against a default apoptotic program. Consistent with earlier reports, we show that potent inhibition of survivin expression by LY2181308-induced caspase-3 activity in HeLa and other tumor cell lines.

We show that LY2181308 reproducibly inhibited survivin protein expression in at least two different xenograft models when administered intravenously. On the basis of the kinetics study after a single-dose administration of LY2181308 into tumor bearing nude mice it is clear that inhibition of survivin expression peaked between 24 and 48 hours. We were unable to see significant survivin inhibition beyond 48 hours. This indicates the need to administer subsequent doses of the compound approximately every 48 hours to maintain inhibition of survivin protein expression. Indeed this is true as we clearly show inhibition of survivin expression in xenograft tumors when animals were treated with LY2181308 every other day for 2 weeks. More importantly, inhibition of survivin expression is associated with
significant reduction in the tumor size when compared with vehicle and MM control–treated groups. Although average percentage inhibition of survivin expression in xenograft is modest, we attribute several factors influencing this magnitude. These includes, uneven exposure of ASOs in tumor, and dilution of inhibitory effect with survivin levels from cells not taking up ASOs as entire tumor gets homogenized for immunoblotting analysis. Furthermore, histologic studies with tumors excised from animals receiving multiple doses of LY2181308 show the expected phenotypic effects of survivin inhibition. We showed for the first time that LY2181308 treatment induced accumulation of multinucleated cells and increased cyclin B1 expression in xenograft tumors, phenotypes that are consistent with inhibition of survivin function. Interestingly, cyclin B1 accumulation remained mostly in the cytoplasm. It is quite possible that this may be due to timing of the tumor harvest. Long-duration treatment of xenograft tumor bearing animals with LY2181308 produced significant antitumor activity that was dose-dependent. We provide evidence that inhibition of survivin expression by LY2181308 sensitizes HeLa cells to gemcitabine and paclitaxel in a cell culture setting as measured by caspase-3 activity that was greater than additive. Importantly, LY2181308 treatment sensitized xenograft tumors to gemcitabine, paclitaxel, and docetaxel. As determined by statistical analysis, the combination effect of LY2181308 with gemcitabine or paclitaxel was synergistic, and the combination effect of LY2181308 with docetaxel was better than either single agent. Because overexpressed survivin is proposed to block default apoptosis in the cells, it is not surprising to see a massive amount of apoptosis and tumor regression. One would expect restoration of default apoptotic pathway due to inhibition of survivin expression. Although LY2181308 alone or in combination with gemcitabine or paclitaxel failed to produce tumor regression in these xenograft studies, combination with other targeted agents with specific apoptosis inducing mechanism may provide improved efficacy. Furthermore, optimization of dosing sequence in combination studies may also enhance efficacy.

In summary, because of its differential expression in tumors versus normal tissues and its role in apoptosis for maintaining cell viability and in cell division, survivin represents a promising drug target for cancer therapy. However, conventional therapeutic intervention approaches to inhibit survivin function remain challenging. For the very first time, we provide evidence that survivin expression and its role in cell cycle, cell division, and apoptosis can be inhibited in the xenograft tumors by intravenous administration of LY2181308, which translates into inhibition of xenograft tumor growth and sensitization of tumors to chemotherapeutic agents. These important preclinical data provided a foundation in support of clinical development of LY2181308 for the treatment of survivin positive human cancers. Because of the specificity nature of ASO, it is expected that LY2181308 would be most effective in patients with tumor over-expressing survivin, thus providing an option to tailor this agent for individual patient based on the survivin expression status. Furthermore, based on tissue distribution studies it is known that concentration of oligonucleotides are generally high in certain tissues such as kidney, liver, spleen, lymph nodes, bone marrow, ovary, testis, pancreas, and lung (49). This tissue distribution pattern provides greater access of ASOs to its target and thus may provide therapeutic benefits to patients with malignancies of these tissues. The LY2181308 in combination with docetaxel is currently being evaluated in a Phase II clinical trial of prostate cancer.

Disclosure of Potential Conflicts of Interest

P. Iversen and B.K.R. Patel are employees of Eli Lilly and Company. E. Marcusson is an employee of ISIS Pharmaceuticals Inc.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received September 1, 2010; revised November 11, 2010; accepted December 4, 2010; published OnlineFirst January 7, 2011.

References

13. Bianc-Brude OP, Mesri M, Wall NR, Plescia J, Dohi T, Altiere DC. Therapeutic targeting of the survivin pathway in cancer: initiation of...


Molecular Cancer Therapeutics

Antisense Inhibition of Survivin Expression as a Cancer Therapeutic
Rosa A. Carrasco, Nancy B. Stamm, Eric Marcusson, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-10-0756

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2011/01/07/1535-7163.MCT-10-0756.DC1

Cited articles
This article cites 47 articles, 12 of which you can access for free at:
http://mct.aacrjournals.org/content/10/2/221.full#ref-list-1

Citing articles
This article has been cited by 2 HighWire-hosted articles. Access the articles at:
http://mct.aacrjournals.org/content/10/2/221.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.