Cantharidin-induced mitotic arrest is associated with the formation of aberrant mitotic spindles and lagging chromosomes resulting, in part, from the suppression of PP2Aα

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Abstract
Cantharidin, a natural vesicant, inhibits the activity of several PPP family phosphatases, displays antitumor activity, and induces apoptosis in many types of tumor cells. However, the molecular mechanisms underlying the antitumor activity of cantharidin are not clear. Here, dose-response studies confirm a strong correlation between the suppression of phosphatase activity and cell death. Flow cytometry analysis indicates that before apoptosis, cantharidin delays cell cycle progression following DNA replication with no apparent effect on G1-S or S-G2 phase progression. In contrast, studies with double thymidine-synchronized populations of cells indicate that cantharidin can rapidly arrest growth when added during G2 or early M phase. Immunostaining indicates that cell cycle arrest occurs before the completion of mitosis and is associated with the appearance of aberrant mitotic spindles. Live cell imaging with time-lapse microscopy shows that cantharidin disrupts the metaphase alignment of chromosomes and produces a prolonged mitotic arrest, with the onset of apoptosis occurring before the onset of anaphase. To explore the contribution of individual phosphatases, antisense oligonucleotides and small interfering RNA were developed to suppress the expression of cantharidin-sensitive phosphatases. The suppression of PP2Aα, but not PP2Aβ, is sufficient to induce metaphase arrest, during which time lagging chromosomes are observed moving between the spindle poles and the metaphase plate. Immunostaining revealed slightly abnormal, yet predominately bipolar, mitotic spindles. Nonetheless, after a 10- to 15-hour delay, the cells enter anaphase, suggesting that an additional cantharidin-sensitive phosphatase is involved in the progression from metaphase into anaphase or to prevent the onset of apoptosis in cells arrested during mitosis. [Mol Cancer Ther 2006;5(11):2727–36]

Introduction
Tablets dated to ~180 AD from the Han Dynasty describe the use of Mylabris, a concoction produced from the Chinese blister beetle, for the treatment of abdominal mass (1). Therefore, Mylabris represents one of the oldest treatments for cancer in recorded history. In 1810, a French pharmacist (Robiquet) developed a procedure to produce an active constituent of Mylabris, cantharidin (exo, exo-2,3-dimethyl-7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid anhydride; Fig. 1), which was followed by a period in which cantharidin was employed as an antitumor drug in Europe. By the early 1900s, however, cantharidin was considered by most physicians as too toxic for internal use and fell out of favor as a treatment for human cancer (2). Today, Mylabris is still employed in traditional Chinese medicine, and, recently, interest in the clinical use of cantharidin as an antitumor agent has re-emerged following reports that cantharidin and synthetic derivatives produce cytotoxic effects in a number of human tumor cell lines. Cantharidin also has cytotoxic activity against a number of primary tumor cells (3–7), and clinical testing revealed little myelosuppression (1, 3). Still, the clinical usefulness of cantharidin is limited by renal and mucous membrane toxicity (3, 8).

Case studies of human poisonings indicate that at high concentrations, cantharidin acts as an acute toxin, producing nausea, severe abdominal pain, shock, convulsions, coma, and death (2). In cultured cells, when applied at concentrations >10 μmol/L, cantharidin also kills animal cells acutely (<24 hours; ref. 7). Such toxic effects will likely prevent cantharidin from being developed for mainstream oncology. Still, when applied at lower concentrations (~2–5 μM), cantharidin can elicit a strong apoptotic response in many types of tumor cells (9–11), characterized by membrane blebbing, caspase activation, DNA fragmentation (11), phosphorylation/activation of p53, and increased levels of both p21WAF1/CIP1 (10) and Bax (9). Cantharidin treatment has also been shown to produce a...
was then diluted (Tris-HCl, 1 mmol/L EDTA, and 2 mmol/L DTT (pH 7.4]). The homogenate with 4 cantharidin as described in Materials and Methods. phosphatase activity in the presence of the indicated amount of A549 cells. Cells from a single independent phosphatase activity contained in a crude homogenate of proliferation. A, a common catalytic mechanism (17), and structure/activity structurally related PPases (14–16). Recent structural potent inhibitor of three families (PP1, PP2A, and PP5) of acting as a weak inhibitor of calcineurin (PP2B) and a family serine/threonine protein phosphatases (PPases). The studies presented indicate that cantharidin-induced apoptosis is preceded by the formation of aberrant mitotic spindles and metaphase growth arrest. Studies with antisense and siRNA indicate that PP2A is needed for proper chromosome segregation, and that the suppression of PP2A alone is sufficient to produce the prolonged mitotic arrest.

Materials and Methods

Cell Culture

A549 cells were obtained from the American Type Culture Collection (Manassas, VA), and HeLa-H2B-GFP cells were a generous gift from Dr. Kevin Sullivan (The Scripps Research Institute, La Jolla, CA). Both cell lines were cultured in DMEM supplemented with 10% fetal bovine serum and L-glutamine (4.0 mmol/L) at 37°C in 5% CO2.

Phosphatase Assays

The production of phosphohistone and the measurement of phosphatase activity were conducted as described previously (14, 19).

Analysis of Cell Growth and Proliferation Assays

Cells were seeded in 12-well tissue culture plates at a density of 5 x 10^4 per dish. On the next day, the cells were treated with PP2A-specific antisense oligonucleotides or the scrambled mismatch controls at a final concentration of 300 nmol/L as described above. On each of the next 5 days, the cell cultures were treated briefly with trypsin to detach the cells from the dish (three wells from each test group). The number of cells was then determined by counting using a hemacytometer. Cell viability was determined with trypan blue staining. The percentage of viable cells was calculated by dividing the number of cells decrease in the levels of two proteins that suppress apoptosis: bcl-2 and survivin (9). Therefore, understanding the biological actions of cantharidin may provide insight for the development of less toxic analogues that retain antitumor activity.

A key breakthrough in this area came from studies which showed that cantharidin binds with high affinity to a protein (12), which was thereafter identified as a protein phosphatase (13). Subsequent studies revealed that cantharidin functions as an inhibitor of several PP family serine/threonine protein phosphatases (PPases), acting as a weak inhibitor of calcineurin (PP2B) and a potent inhibitor of three families (PP1, PP2A, and FPS) of structurally related PPases (14–16). Recent structural studies revealed that the cantharidin-sensitive PPases share a common catalytic mechanism (17), and structure/activity relationship studies indicate that cantharidin (15) and fostriecin (18), another natural product with antitumor activity, both act to inhibit the catalytic activity of the same PPP family phosphatases (15, 17, 18). Human genetic studies have identified four highly homologous isoforms of PP1 (PP1α, PP1β/δ, PP1γ, and PP1δ), two nearly identical isoforms of PP2A (PP2α and PP2β), and two PPases (designated PP4 and PP6) that, at the level of their primary amino acid sequence, share ~54% and 60% identity with PP2Aα, respectively (for review, see ref. 15). Therefore, humans express at least nine cantharidin-sensitive PPases that are expressed ubiquitously.

To date, the inhibitory actions of cantharidin against purified phosphatases is well documented (15), and several studies indicate that cantharidin is capable of eliciting a “classic” apoptotic response. Nonetheless, little is known about the molecular mechanisms by which the inhibition of phosphatase activity induces or promotes a cytotoxic/antitumor effect, how the apoptotic/cytotoxic response is initiated, or even which of the nine cantharidin-sensitive phosphatases are involved. Here, we further characterize the biological activity of cantharidin in culture cells (A549 and HeLa) and develop small interfering RNA (siRNA) and antisense oligonucleotides capable of markedly suppressing the expression of several cantharidin-sensitive phosphatases. The studies presented indicate that cantharidin-induced apoptosis is preceded by the formation of aberrant mitotic spindles and metaphase growth arrest. Studies with antisense and siRNA indicate that PP2Aα is needed for proper chromosome segregation, and that the suppression of PP2Aα alone is sufficient to produce the prolonged mitotic arrest.

Figure 1. Cantharidin inhibits PPP family phosphatases and cell proliferation. A, effect of cantharidin (inset) on the divalent cation-independent phosphatase activity contained in a crude homogenate of A549 cells. Cells from a single ~70% confluent 100-mm dish were rinsed with 4°C PBS, scraped, and sonicated in 1 mL Tris buffer (20 mmol/L Tris-HCl, 1 mmol/L EDTA, and 2 mmol/L DTT (pH 7.4)). The homogenate was then diluted (~65 µg protein/mL) and assayed for phosphohistone phosphatase activity in the presence of the indicated amount of cantharidin as described in Materials and Methods. B, effect of cantharidin on cell viability. A549 cells were plated in 96-well plates (5,000 per well) and allowed to grow. After 24 h, the indicated concentration of cantharidin was added, and the cells were incubated for the time indicated at 37°C. Cell viability was then measured using a Cell Titer 96 Aqueous One Solution Cell proliferation Assay according to the methods of the manufacturer (Promega). Points, mean of three independent experiments each conducted in triplicate; bars, mean of three independent experiments each conducted in triplicate; bars, mean ± SD.
excluding trypan blue by the total number of cells, and the results are reported as the mean ± SD of data collected from three independent experiments. The ability of cantharidin to inhibit the growth of cultured cells was determined using a tetrazolium-based proliferation assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and by counting the number of cells daily using a culture counter. Cell death was visualized by trypan blue exclusion and DNA staining using propidium iodide (50 g/mL) and Alexa Fluor 488 antibodies (Invitrogen; secondary mouse monoclonal, 1:1,000). The samples were then examined using a Leica TCS SP2 confocal system equipped with argon, CO2, and Alexa Fluor 594 antibodies (Invitrogen; secondary goat anti-rabbit, 1:1,000). The samples were then examined using a Leica TCS SP2 confocal system equipped with argon, krypton, and He-Ne lasers. Confocal images were collected at 1.0-μm z intervals with a pinhole setting of 80 μm. Typically, 20 optical sections were analyzed per cell.

**Flow Cytometry**

At the time indicated, cells were subjected to a brief treatment with trypsin, collected by centrifugation, resuspended in PBS, and stained with Vindelov’s propidium iodide stain (50 μg/mL) containing RNase (200 μg/mL) at 4°C. DNA content flow cytometry analysis was done using routine methods with a Becton Dickinson fluorescence-activated cell sorter (FACS), gating to exclude cell debris as described previously (20).

**Indirect Immunofluorescence Microscopy**

Cells were plated onto 60-mm dishes containing 22 × 22 coverslips in 3 mL DMEM at a concentration of 300,000 per dish. At timed intervals, the coverslips were removed, washed with PBS (pH 7.4), fixed with methanol at −20°C, permeabilized with Triton X-100, and processed for immunofluorescence microscopy using previously published methods (21). Microtubules were visualized with anti-a-tubulin (Sigma, St. Louis, MO; primary, 1:100 dilution) and Alexa Fluor 488 antibodies (Invitrogen, Carlsbad, CA; secondary mouse monoclonal, 1:1,000). DNA was visualized with propidium iodide (50 μg/mL) or Draq5 (Biostatus Products, Shepshed, United Kingdom; 1:300). Centrosomes were visualized using anti-pericentrin (Covance, Berkeley, CA; primary rabbit polyclonal, 1:500) and Alexa Fluor 594 antibodies (Invitrogen; secondary goat anti-rabbit, 1:1,000). The samples were then examined using a Leica TCS SP2 confocal system equipped with argon, krypton, and He-Ne lasers. Confocal images were collected at 1.0-μm z intervals with a pinhole setting of 80 μm. Typically, 20 optical sections were analyzed per cell.

**Live Cell Imaging and Time-lapse Video Microscopy**

Cells were cultured in 60-mm dishes and incubated at 37°C in 5% CO2 using a Neave live cell chamber fitted into an Eclipse Nikon TE 2000-U microscope (Nikon Instruments, Inc., Melville, NY). Image acquisition was achieved using a COOLSNAP ES monochrome camera and processed with MetaMorph Premier software (Universal Imaging, Downingtown, PA).

**Oligonucleotide Synthesis and Assay for Oligonucleotide Inhibition of PP2Aα Expression**

Phosphorothioate deoxyoligonucleotides and 2′-O-methyl phosphorothioate deoxyoligonucleotides were synthesized and purified as described previously (22–24). The indicated cells were plated in 60-mm dishes and cultured in DMEM containing 10% FCS. When the cells were ~50% confluent, they were treated with oligonucleotides as previously described (22, 23). After treatment (4 hours), the cells were washed and cultured in fresh DMEM containing 10% FCS for 17 hours. The cells were then harvested, and total RNA was extracted with TRIzol Reagent (Life Technologies, Gaithersburg, MD) according to the methods of the manufacturer. Total RNA (20 μg) was fractionated on 1% agarose gels containing formaldehyde and transferred to DURILON-UV (Stratagene, Cedar Creek, TX) nylon membranes. Following UV cross-linking, the filters were hybridized with a [32P] probe for human PP2Aα or PP2Aβ as indicated. The human PP2A cDNA probes were generated from the full-length coding region of PP2Aα or PP2Aβ, respectively, and [32P] labeled with a DECAprime DNA Labeling kit (Ambion, Austin, TX) according to the manufacturer’s protocol. Hybridization was done in the presence of 50% formamide at 42°C for 16 hours. Following hybridization, the membrane was subjected to two low-stringency washes (2 × SSC) at room temperature and then two high-stringency washes (0.1 × SSC/0.5% SDS) at 55°C. Hybridization was visualized by autoradiography, and quantification of hybridization signals was obtained using a Fuji FLA-5000 imaging system. The membranes were then stripped and reprobed with a [32P] labeled glyceraldehyde-3-phosphate dehydrogenase cDNA probe to confirm that equal loading was achieved. Quantification of probe binding was achieved using a Fuji FL5000 imaging system.

**Immunoblotting of PP2A**

Western analysis was done essentially as described previously using polyclonal rabbit antibodies generated against a synthetic 15-amino-acid peptide identical to the COOH-terminal region of PP2A that does not discriminate between PP2Aα and PP2Aβ (25).

**Results**

**Cantharidin-Induced Cytotoxicity Correlates with the Inhibition of Divalent Cation-Independent Phosphatase Activity**

Using a [32P]phosphohistone-based phosphatase assay, the divalent cation-independent serine/threonine phosphatase activity contained in dilute human cell homogenates represents the activity of PP1, PP2A, PP4, PP5, and possibly PP6 (15). The IC50 (concentration inhibiting 50% activity) for cantharidin-mediated inhibition of divalent cation-independent PPase activity in A549 cell homogenates was estimated at ~0.5 μmol/L (Fig. 1A). To compare PPase inhibition with growth suppression and cytotoxicity, dose-response studies were conducted in which replicate plates were treated with the indicated concentrations of cantharidin. At timed intervals, cell proliferation and viability were assessed using a metabolic assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay], a cell proliferation assay (counting the number of cells using a culture counter), and a trypan blue exclusion assay (counting the number of live cells based on their ability to...
exclude trypan blue). All three of these methods produced complementary results (Fig. 1B). At high concentrations (>10 μmol/L), cantharidin rapidly inhibits proliferation, and trypan blue exclusion studies indicate that >95% of the cells were killed in <24 hours. In contrast, treatment with low concentrations (<0.1 μmol/L) for up to 72 hours had no apparent effects on cell viability, growth, or morphology. At concentrations sufficient to inhibit the activity of PP1-PP5 (1–5 μmol/L), cell-counting studies indicate that cantharidin rapidly (~24 hours) inhibits cell proliferation in a dose-dependent manner. Viability studies indicate that by ~48 to 72 hours, most of the cells treated with >2 to 5 μmol/L cantharidin die (Fig. 1B). For A549 cells, the apparent LC50 (concentration killing 50% of the cells) for continuous exposure at 48 hours was ~2.0 μmol/L, which correlates with the near complete inhibition of divalent cation-independent PPase activity (i.e., PP1–PP5).

**Cantharidin Treatment Increases the Proportion of Cells in the G2-M Phase of the Cell Cycle**

To further characterize the growth arrest induced by cantharidin, A549 cells were treated with the indicated amount of cantharidin (0–5 μmol/L) and then subjected to FACS analysis. FACS analysis of propidium iodide–stained cells indicates that the number of cells containing...
twice the $G_1$ content of DNA (4N) increases in a dose-dependent manner 24 hours after treatment with 2 to $5 \mu$mol/L cantharidin (Fig. 2A). To further test the effects of cantharidin on the cell cycle progression, A549 cell cultures that were synchronized by double thymidine treatment were treated with cantharidin immediately after release from thymidine-induced growth arrest. As seen in Fig. 2B, following release from the second thymidine block, control cells rapidly progress into S phase, and cells with 4N DNA are observed after ~6 to 7 hours. The cells then divide, and <12 hours after release, a $G_1$ population of cells is again observed. When the double thymidine arrested cells were treated with cantharidin immediately after release from growth suppression, the progression of cells into S phase was not delayed (compare Fig. 2B and C; 4 HR). However, following DNA replication, the majority of the cantharidin-treated cells arrest, most still containing 4N DNA 16 hours after treatment (compare Fig. 2B and C; 12 and 16 HR).

When cantharidin was added to double thymidine-arrested cell cultures at timed intervals (2, 4, 6, and 7 hours after release from $G_1$ growth suppression), FACS analysis showed that all treatment groups contained 4N DNA at 16 hours after release (Fig. 2D). These studies suggest that cantharidin-induced growth arrest occurs after DNA replication is complete but before cell division (i.e., $G_2$-$M$ phase).

**Cantharidin Treatment Induces Abnormal Mitotic Spindle Formation**

To explore mechanisms associated with cantharidin-induced $G_2$-$M$ phase growth arrest, we examined the effects of cantharidin on microtubule behavior. Immunostaining with anti-α-tubulin antibodies revealed marked differences between the control and cantharidin-treated cells (Fig. 3). At mitosis, >98% of the control cells contain a typical array of anti-parallel microtubules characteristic of a normal bipolar mitotic spindle (Fig. 3A). In contrast, in the cantharidin-treated cells (Fig. 3B–D), the condensed chromosomes were not aligned at the metaphase plate, and the spindle apparatus was aberrant, often composed of multiple “bundles” (Fig. 3E). The percentage of cells containing aberrant spindles increased in a dose-dependent manner (Fig. 3F), and based on observations of mitotic structures by confocal fluorescence microscopy, it seems that cantharidin does not prevent the polymerization of microtubule structures in general. Rather, cantharidin disrupts the organization of the spindles. In nondividing cells, there was no apparent difference in the microtubules in control and cantharidin-treated cultures (data not shown).

**Development and Characterization of Antisense Oligonucleotides Capable of Suppressing the Expression of Human PP2A**

Because the amount of cantharidin needed to induce growth arrest is sufficient to markedly inhibit the activity of all isoforms of PP1, PP2A, PP4, and PP5, it was not clear which of the cantharidin-sensitive phosphatase(s) is needed for progression through mitosis. Therefore, to further characterize the roles of PPases in cell cycle progression, we developed siRNA and antisense oligonucleotides to

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**Figure 3.** Abnormal spindle formation in cells treated with cantharidin. Representative A549 cells treated with solvent alone (A) or cantharidin (B–E). A549 cells were treated with 2 μmol/L cantharidin and fixed on coverslips 24 h later. Microtubules (green) were then visualized by immunofluorescence following treatment with anti-α-tubulin (primary) and Alexa Fluor 488–labeled (secondary) antibodies. DNA (red) was visualized by staining with propidium iodide. E, serial z sections obtained by confocal microscopy of cantharidin-treated cells stained for anti-α-tubulin antibodies as above. F, effect of cantharidin on spindle formation. A549 cells were treated with the indicated amount of cantharidin. After 24 h, the cells were fixed. Microtubules and DNA were then visualized as described above. Metaphase cells (300 for each concentration of cantharidin) were then scored as containing normal or aberrant spindles. Percentage containing normal bipolar spindles (white columns) or aberrant spindles (filled columns). A to F, similar results were obtained with four or more independent experiments.
potently suppress the expression of human PP2Aα and PP2Aβ, the two PP2As determined to be highly sensitive to cantharidin using a phosphoprotein-based phosphatase assay (i.e., the PP2As with IC₅₀ < 2 μmol/L). For these studies, ∼45 different oligonucleotides targeting unique sequences contained in the mRNA of the respective PP2As were synthesized and tested. Each antisense oligonucleotide tested was 20 bases in length and designed to hybridize to a unique region of human PP2Aα or PP2Aβ. Twenty-three were designed to target PP2Aα and 20 targeted sequences unique to PP2Aβ (Fig. 4A). All of the oligonucleotides tested in the primary screen were second-generation “chimeric” oligonucleotides, containing 10 central phosphorothioate oligodeoxy residues (“gap”) flanked by five to six 2'-methoxyethyl-modified residues on the 3’ and 5’ ends. These modifications have been shown previously to enhance the potency of antisense oligonucleotides targeting mRNAs encoding other proteins (22, 23). Because phosphorothioate oligonucleotides have been shown to commonly act through RNase H-dependent mRNA cleavage mechanism in cells (26), the ability of each oligodeoxynucleotide to specifically inhibit the expression of PP2A was initially determined by Northern blot analysis.

Probing for levels of PP2A mRNA. PP2A mRNA was detected using PP2Aα- or PP2Aβ-specific cDNA probes, which forms a hybrid with a single transcript of the target mRNA.

A comparison of PP2A mRNA levels in A549 human lung carcinoma cells treated with 300 nmol/L PP2Ac-specific antisense oligonucleotides in the presence of cationic lipids is shown in Fig. 4. The cationic lipids (DOTMA/DOPE; Lipofectin) were used to facilitate the uptake of the oligonucleotides (24, 27, 28), and the reduction in PP2A mRNA levels observed in response to treatment was varied. Treatment with some oligonucleotides had little or no effect on the inhibition of PP2A mRNA levels, whereas others had a moderate effect, and a few had pronounced effects (Fig. 4). The antisense oligonucleotide with the most potent activity against PP2Aα mRNA identified in this series was ISIS 110159 (IC₅₀ ~ 18 nmol/L; Fig. 4C), which targets a region contained in the coding region of PP2Aα. The antisense oligonucleotides with the most potent activity against PP2Aβ mRNA identified in this series were ISIS 110179, ISIS 110181, and ISIS 110186 (Fig. 4B), which target two regions contained in the coding region and one region contained in the 5’-untranslated region of PP2Aβ. The

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Figure 4. Inhibition of PP2A mRNA and protein expression by treatment with antisense oligodeoxynucleotides. A, relative positioning of the predicted hybridization sites within the human PP2Aα or PP2Aβ mRNA of 43 antisense oligodeoxynucleotides that were evaluated for their ability to inhibit PP2A expression in cultured A549 cells. Antisense oligodeoxynucleotides that inhibit the expression of PP2Aα and PP2Aβ mRNA were identified by treating A549 cells with the indicated oligodeoxynucleotides at a concentration of 300 nmol/L. mRNA was prepared 24 h later and analyzed for PP2Aα, PP2Aβ, and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA levels by Northern blot analysis. B, inhibition of PP2Aβ mRNA levels by ISIS 110179, ISIS 110181, ISIS 110186, or ISIS 110189. C, inhibition of PP2Aα by ISIS 110159 or ISIS 110163. D, specificity of oligos targeting PP2Aα (ISIS 110159) and PP2Aβ (ISIS 110181). A549 cells were treated with increasing concentrations (0–300 nmol/L) of the indicated antisense oligodeoxynucleotides or corresponding mismatched control analogues (MM) that contain 13 base changes (mismatches) within the sequence of the indicated target. Total mRNA was prepared 24 h later and analyzed for PP2Aα, PP2Aβ, and G3PDH mRNA levels by Northern blot analysis. IC₅₀s were estimated from plots produced by quantification of PP2A mRNA levels after normalization to G3PDH mRNA in A549 cells following treatment with increasing concentrations of oligodeoxynucleotides (illustrated for ISIS 110159). E, Western blots of PP2A protein levels in A549 cells. Cells were treated with the mismatch control oligodeoxynucleotides, ISIS 110189 (targeting PP2Aα) or ISIS 110181 (targeting PP2Aβ), and protein extracts were prepared 48 or 76 h later. Each lane contained 40 μg protein. F, target-specific inhibition of ISIS 110159 and ISIS 110181. A549 cells were treated with 200 or 400 nmol/L antisense oligodeoxynucleotides targeting the indicated isoform of PP2A. Total protein was prepared 48 or 76 h later and analyzed for PP4 and PP6 by Western blot analysis.
effective oligonucleotides and several of the oligonucleo-
tides with moderate or no effect were also tested against
other human cell lines for effects on the expression of PP2A
mRNA levels with essentially identical results.

Specificity of Antisense Inhibition

To examine the specificity of oligonucleotides targeting
PP2Aα (ISIS 110159 and ISIS 110163) and PP2Aβ (ISIS
110179, ISIS 110181, ISIS 110186, and ISIS 110189),
the effects of each oligonucleotide and appropriate mismatch
control analogues (designated ISIS 110159 MM1, etc.)
were tested. The mismatch control analogues contain the
same base composition as the corresponding antisense
oligonucleotide but with sequences noncomplementary
to the target mRNA. As seen in Fig. 4B to E, treatment of
A459 cells with the indicated oligonucleotides produced a
dose-dependent reduction of the appropriate PP2A mRNA
levels with the most potent antisense oligonucleotides
having IC$_{50}$ < 50 nmol/L. No effect was observed
following treatment with the mismatch controls (Fig. 4D),
even when applied at concentrations 10 times that of
the IC$_{50}$ for highly active oligonucleotides. Blots were
stripped and reprobed with a glyceraldehyde-3-phosphate
dehydrogenase cDNA probe to show equal RNA loading.
Studies with additional mismatch controls and different
cell types produced similar results (data not shown).

Although the differences in codon usage allowed for the
production of isoform-specific probes suitable for Northern
analysis, PP2Aα and PP2Aβ are 97% identical at the level
of their primary amino acid sequences. Therefore, it was
not possible to generate isoform-specific antibodies. None-
theless, Western analysis using an antibody that recognizes
both isoforms of PP2A revealed that treatment with ISIS
110159 (targeting PP2Aα) decreased PP2A levels by 80%
after ~48 hours (Fig. 4E). The suppression of PP2Aβ with
ISIS 110181 produced an ~20% decrease. Treatment with
200 nmol/L of ISIS 110159 in combination with 200 nmol/L
ISIS 110181 produced a marked suppression of PP2A after
48 hours. These observations are consistent with reports
studying the expression of PP2A isoforms, in which PP2Aα
mRNA levels were observed at approximately four times
the level of PP2Aβ.

To further test the specificity of the antisense oligonu-
cleotides targeting PP2A mRNA, the effect of ISIS 110159
on expression of two structurally related PPases (PP4
and PP6) was tested by Western analysis using antibodies
generated against peptides unique to the indicated
PPases. As seen in Fig. 4F, ISIS 110159 has no effect on
the expression of PP4 or PP6, even at a concentration
(400 nmol/L) that is >20 times the IC$_{50}$ (17.8 nmol/L) for
PP2Aα. Because the sequence targeted by ISIS 110159 is
not contained in PP4 or PP6, ISIS 110159 would not be expected
to inhibit the expression of these proteins if ISIS 110159 is
inhibiting the expression of PP2A mRNA via an RNAse
H-mediated antisense mechanism.

Reduction of PP2Aα Expression with ISIS 110159
Leads to Failure of Various Mitotic Events

FACS analysis of propidium iodide–stained A549 cells
revealed that the suppression of PP2Aα with ISIS 110159
produced an increase in cells containing 4N DNA that
was comparable with cells 24 hours after treatment with
2 μmol/L cantharidin. In contrast, FACS analysis of cells
treated with oligonucleotides targeting PP2Aβ or PP5 failed
to show an increase in cells containing 4N DNA (data not
shown). Immunostaining with anti-α-tubulin antibodies
revealed differences in the appearance of the mitotic
spindles between control and cantharidin or ISIS 110159
(targeting PP2Aα)–treated cells (compare Figs. 3 and 5).
As observed following treatment with cantharidin, cells
treated with ISIS 110159 contained condensed chromo-
somes, and immunostaining with antibodies targeting
pericentrin revealed the normal complement of two
centrosomes in control, cantharidin-treated (data not
shown) and ISIS 110159–treated cells A549 cells (Fig. 5B).
In addition, the mitotic spindles in ISIS 110159–treated
metaphase cells were not well organized. However, most
cells still produced a somewhat bipolar spindle apparatus
(Fig. 5E). As observed with cantharidin, the microtubules in
nondividing cells with suppressed PP2Aα expression were
similar in appearance to untreated or mismatched controls
(data not shown).

To further characterize the cellular effects of cantharidin
and ISIS 110159, live cell imaging in combination with time-
lapse video microscopy was employed using a HeLa cell
line that stably expresses histone-2B fused to enhanced
green fluorescent protein (H2B-GFP). This cell line consti-
tutively expresses H2B-GFP and has been shown to allow
high-resolution imaging of chromosomes without
compromising nuclear and chromosomal structures (29).
In untreated HeLa-H2B-GFP cells, the progression from
chromosome condensation to cytokinesis requires ~2 to
2.5 hours, with metaphase alignment occurring ~20
minutes after chromosomal condensation is first apparent,
and the onset of anaphase occurring ~50 minutes
thereafter (Fig. 6A). In comparison, when HeLa-H2B-GFP
cells are treated with cantharidin, although no apparent
differences from controls were observed before chromo-
some condensation, the condensed chromosomes fail to
align at the metaphase plate and cell cycle progression is
delayed for ~12 to 15 hours (Fig. 4B). During this period,
the cells remain rounded in appearance, and the chromo-
somes remain condensed. After ~12 to 15 hours, the
“arrested” cells become apoptotic.

When HeLa-H2B-GFP cells are treated with cantharidin
after the chromosomes are already aligned at the meta-
phase plate (Fig. 6C), the aligned chromosomes fail to
separate, and alignment is maintained for ~3 hours. The
condensed chromosomes then move away from the
metaphase plate. However, unlike controls in which sister
chromatids move towards the opposite poles of the cell, in
the cantharidin-treated cells, the movement is unorganized
and seems random in nature.

When HeLa-H2B-GFP cells were treated with ISIS 110159,
as observed with other human cell lines, ~30 hours
was required for PP2A protein levels to become suppressed.
Before this time, cell division occurs normally. When
PP2Aα levels were suppressed, time-lapse fluorescence
microscopy indicated that in prometaphase or metaphase cells, most of the chromosomes are condensed and eventually align at the metaphase plate. However, in nearly all instances, anaphase was delayed for >15 hours (Fig. 6D). ISIS 110159–treated cells also exhibited small populations of chromosomes localized at various points between the metaphase plate and the spindle pole (Fig. 6E, arrows). In addition, whereas the cantharidin-arrested cells fail to enter anaphase and become apoptotic, the ISIS 110159–treated cells eventually enter anaphase and divide after the prolonged metaphase arrest. Still, it should be noted that ~48 to 72 hours after treatment with ISIS 111059, numerous apoptotic cells were observed.

Discussion
Here, as observed in T24 cells (7), with A549 and HeLa cells, we observed acute toxicity by cantharidin at high concentrations (≥10 μmol/L) and apoptosis at slightly lower concentrations (2–5 μmol/L). As observed in other studies, dose-response studies revealed a strong correlation between the inhibition of divalent cation-independent serine/threonine phosphatase activity (i.e., PP1–PP5) and cell death. In addition, this study shows that before the onset of apoptosis in dividing cells, cantharidin induces a prolonged mitotic arrest that is associated with the formation of aberrant mitotic spindles, the failure of condensed chromosomes to align at the metaphase plate, and the failure of cells to enter anaphase (Figs. 1–3). Time-lapse microscopy studies also indicate that the cells that arrest in metaphase become apoptotic after a prolonged (>10 hours) delay. In contrast, the nondividing cells are similar in appearance to controls.

To gain insight into which of the cantharidin-sensitive PPases affect cell cycle progression and survival, we developed siRNA and antisense oligonucleotides capable of suppressing the expression of PP1γ1 (20), PP5 (24, 28, 30), and here, PP2Aα and PP2Aβ. FACS analysis of propidium iodide–stained A549 cells treated with siRNA or antisense oligonucleotides targeting each of these PPase individually revealed that the suppression of PP5 expression results in a G1 growth arrest with the concomitant increase in the expression of p21Waf1/cip1 (24). Subsequently, this response was shown to be dependent on the presence of both glucocorticoid receptors and the p53 tumor suppressor protein (24). Moreover, the suppression of PP5 unveiled a link between GR activation, the phosphorylation of p53 at Ser15, and p21-mediated G1 growth arrest (28). Because Ser15 phosphorylation of p53 is known to aid p21 expression, part of the cantharidin-mediated cellular responses can be mimicked by the suppression of PP5 expression. Nonetheless, in cells lines lacking p53 or GRs, the suppression of PP5 expression with antisense or siRNA did not result in an extensive mitotic arrest or prolonged progression into anaphase, suggesting that the inhibition of PP5 alone is not sufficient to induce a cantharidin-like mitotic delay.

Figure 5. Mitotic abnormalities associated with the suppression of PP2Aα. Representative confocal microscopic images of A549 cells treated with ISIS 110159. A549 cells were treated with 300 nmol/L ISIS 110159 and fixed on coverslips 24 h later. A, microtubules (green) were then visualized by immunofluorescence following treatment with anti-α-tubulin and Alexa Fluor 488–labeled antibodies as described in Fig. 3. B, centrosomes were visualized by treatment with pericentrin. C, DNA (blue) was visualized by staining with Draq5. D, computer-assisted merge of images (A), (B), and (C). E, representative images of serial z sections of ISIS 110519–treated mitotic cells stained for anti-α-tubulin. Similar results were obtained with five or more independent experiments.
The suppression of PP2Aβ had no apparent effect on cell cycle progression, whereas the suppression of either PP1γ1 or PP2Aα resulted in the accumulation of 4N cells. Studies with antisense targeting PP1γ1 suggest that it is not needed for mitotic spindle formation or progression from metaphase to anaphase. Rather, PP1γ1 is needed in late-stage cytokinesis and the formation of dikaryons (20).

To date, the molecular events affected by PP1γ1 are not clear. Nonetheless, because cantharidin-treated cells arrest in metaphase and become apoptotic before any indications of cytokinesis, PP1γ1 does not seem to contribute to the “cantharidin phenotype” observed in the cell lines studied. In contrast, the data obtained with ISIS 110159 indicate that PP2Aα elicits the failure of several mitotic events that are also induced by 2 to 5 μmol/L cantharidin (a concentration sufficient to completely inhibit PP2A activity; ref. 14). Notably, treatment with ISIS 110159 produces a prolonged mitotic arrest that is associated with aberrant mitotic structures. Still, PP2Aα suppression does not entirely mimic the actions of cantharidin. In comparison with cells treated with ISIS 110159, the spindles of the cantharidin-treated cells are less organized, and the condensed chromosomes do not align at the metaphase plate. In addition, after a 10- to 12-hour delay, the ISIS 110159–treated cells progress into anaphase and divide, whereas the cantharidin-treated cells do not enter anaphase and become apoptotic.

During mitosis, the sister chromatids (replicates of each chromosome) are physically linked before their segregation. This association, called cohesion, must be sustained during the G2 phase of the cell cycle and during most of metaphase because the kinetochores of each sister chromatid must bind microtubules from opposite spindle poles to ensure segregation away from each other at anaphase (31). Sister chromatid cohesion is mediated by a...
complex of proteins called cohesin. In mammals, the phosphorylation of centromeric cohesin during mitosis promotes its dissociation from the chromatids, which is an important part of the “trigger” for chromosome segregation. Therefore, it is critical for cells to guard centromeric cohesion from the actions of mitotic kinases before the onset of anaphase. Recently, PP2A has been shown to associate with Shugosin (Sgo1; Drosophila, MEI-S332), one of the proteins that is needed for the protection of centromeric cohesion in animals (32, 33). In addition, the association of PP2A with Sgo1 has been shown to be necessary for the centromeric localization of Sgo1 and proper chromosome segregation in human cells (32, 33). The data shown here are consistent with the role of PP2A in the protection of chromosome segregation, for cantharidin treatment of cells results in the formation of lagging sister chromatids, our results in the formation of lagging sister chromatids, our manner. Furthermore, because treatment with ISIS 110159 results in the dissociation of the chromosomes aligned on the metaphase plate in a random and unorganized manner. Furthermore, because treatment with ISIS 110159 results in the formation of lagging sister chromatids, our data indicate the involvement of the α isoform of PP2A in the protection of chromosome segregation. Still, additional studies will be needed to elucidate details of how the suppression of PP2Aα results in arrest during metaphase.

In conclusion, PP2Aα is needed for proper chromosome segregation, and the suppression of PP2Aα with ISIS 110159 is sufficient to induce a prolonged metaphase arrest. However, whereas cantharidin produced a similar metaphase arrest that resulted in cell death before anaphase, the suppression of PP2Aα expression did not ultimately prevent cells from entering anaphase. In addition, the mitotic spindles of the cantharidin-treated cells were generally multipolar and less organized than the cells treated with ISIS 110159. Therefore, additional cantharidin-sensitive PPases are likely to also contribute to the cytotoxic actions of cantharidin.

References

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