AS1411 Alters the Localization of a Complex Containing Protein Arginine Methyltransferase 5 and Nucleolin

Yun Teng, Allicia C. Girvan, Lavona K. Casson, William M. Pierce, Jr., Mingwei Qian, Shelia D. Thomas, and Paula J. Bates

Abstract

AS1411 is a quadruplex-forming oligonucleotide aptamer that targets nucleolin. It is currently in clinical trials as a treatment for various cancers. We have proposed that AS1411 inhibits cancer cell proliferation by affecting the activities of certain nucleolin-containing complexes. Here, we report that protein arginine methyltransferase 5 (PRMT5), an enzyme that catalyzes the formation of symmetrical dimethylarginine (sDMA), is a nucleolin-associated protein whose localization and activity are altered by AS1411. Levels of PRMT5 were found to be decreased in the nucleus of AS1411-treated DU145 human prostate cancer cells, but increased in the cytoplasm. These changes were dependent on nucleolin and were not observed in cells pretreated with nucleolin-specific small interfering RNA. Treatment with AS1411 altered levels of PRMT5 activity (assessed by sDMA levels) in accord with changes in its localization. In addition, our data indicate that nucleolin itself is a substrate for PRMT5 and that distribution of sDMA-modified nucleolin is altered by AS1411. Because histone arginine methylation by PRMT5 causes transcriptional repression, we also examined expression of selected PRMT5 target genes in AS1411-treated cells. For some genes, including cyclin E2 and tumor suppressor ST7, a significant up-regulation was noted, which corresponded with decreased PRMT5 association with the gene promoter. We conclude that nucleolin is a novel binding partner and substrate for PRMT5, and that AS1411 causes relocalization of the nucleolin-PRMT5 complex from the nucleus to the cytoplasm. Consequently, the nuclear activity of PRMT5 is decreased, leading to derepression of some PRMT5 target genes, which may contribute to the biological effects of AS1411. [Cancer Res 2007;67(21):10491–500]

Introduction

Oligonucleotide aptamers are short sequences of DNA or RNA (or modified versions thereof) that can bind to specific proteins via recognition of their three-dimensional structure. Thus, they are mechanistically similar to therapeutic monoclonal antibodies, but may have certain advantages over antibodies, such as stability, ease of manufacture, and nonimmunogenicity (1). Aptameric oligonucleotides frequently contain secondary structure motifs, such as hairpins or G-quartets, and are usually discovered by in vitro evolution techniques (1), although some have been discovered by chance (2, 3).

We have previously reported on phosphodiester G-rich oligonucleotides, termed GROs, which function as nucleolin-binding aptamers (3–7). These have strong growth-inhibitory activity against various types of cancer cells, but have minimal effects on nonmalignant cells (4, 7). Active GROs can form stable G-quadruplex structures that are resistant to degradation by heat or exonucleases (5, 6). One of the GROs, now known as AS1411 (formerly AGRO100 or GRO26B-OH), is the first aptamer to be tested in clinical trials as a cancer therapeutic. The results of phase I clinical trials in patients with advanced cancer were presented recently and indicate that AS1411 was well tolerated with no reports of any serious adverse events (8, 9). Furthermore, there was evidence of promising clinical activity, including objective responses in patients with metastatic renal cell carcinoma (8, 9).

Nucleolin (the molecular target of AS1411) is a remarkably multifunctional protein that can be present in the nucleoli, nucleoplasm, cytoplasm, and plasma membrane of cells (reviewed in refs. 10–12). Levels of nucleolin are known to correlate with the rate of cellular proliferation, being elevated in rapidly dividing cells, such as malignant cells, but barely detectable in quiescent cells (13, 14). Some of the most studied aspects of nucleolin biology are its roles in ribosome biogenesis, which include the control of rDNA transcription, preribosome packaging, and organization of nucleolar chromatin. Another major role is as a shuttle protein that transports viral and cellular proteins between the cytoplasm and nucleus/nucleolus of the cell. Nucleolin has also been implicated, directly or indirectly, in many other functions including apoptosis, nuclear matrix structure, DNA replication, mRNA stability, transcriptional regulation, signal transduction, telomere maintenance, cytokinesis, as a nucleic acid helicase, and as a G-quadruplex binding protein (see refs. 3, 4, 7, 10–12, 15 and references therein). In addition, there are numerous reports describing the presence of nucleolin on the cell surface and its function as a receptor for a variety of ligands (16–22). The significance of nucleolin in cancer biology is becoming increasingly apparent and several recent studies have indicated a direct role in malignant transformation (22–26).

Although the primary target of AS1411 has been identified, the precise mechanism of its antiproliferative activity is not yet fully understood. Like many other nucleolin-binding ligands, AS1411 binds to cell surface nucleolin and is internalized by cancer cells. We propose that, once inside the cell, binding of AS1411 modulates the interactions between nucleolin and its binding partners, leading to pleiotropic biological effects. Our current research aims to identify nucleolin-containing complexes that are altered in AS1411-treated prostate cancer cells and to investigate the
biological consequences of these changes. In this report, we describe our identification of protein arginine methyltransferase 5 (PRMT5) as a novel binding partner for nucleolin and the effects of AS1411 on the subcellular localization and activity of PRMT5.

**Materials and Methods**

**Materials.** The human prostate cancer cell line DU145 was obtained from American Type Culture Collection (ATCC). The oligodeoxynucleotides used were AS1411, 5′-d(GGTGTTGGTTGTGTTGGTTGGTT)-3′, and an inactive control oligonucleotide, 5′-d(CCTCCTCTCTCTCTCTCTCT)-3′. Both had a phosphodiester backbone and were purchased in the desalted form from Integrated DNA Technologies. Anti-FLAG peptide antibody (M2), M2-conjugated agarose beads, FLAG peptide, nonionic detergent IGEPA L CA-630, anti–j-actin, and anti-PRMT5 monoclonal antibodies were from Sigma-Aldrich. Anti–asymmetrically dimethylated arginine (aDMA; ASYM24) and anti–asymmetrically dimethylarginine (sDMA; SYM10) polyclonal antibodies were obtained from Upstate Biotechnology. MagnaBind goat anti-mouse IgG beads and anti-rabbit IgG beads were from Pierce. Anti-nucleolin monoclonal antibody (MS-3) and anti-mouse or anti-rabbit antibodies linked to horseradish peroxidase were purchased from Santa Cruz Biotechnology. Mouse IgG beads and anti-rabbit IgG beads were from New England Biolabs.

**Plasmid construction.** To construct FLAG-tagged nucleolin expression plasmid (pFLAG-nucleolin), the NraI-XhoI fragment (encoding amino acids 7–707) of pBS-nucleolin (IMAGE 591D39, ATCC) was subcloned into the pCMV2-FLAG vector (Sigma-Aldrich). The reading frame and sequence of the resulting pFLAG-nucleolin plasmid were confirmed by automated sequencing.

**Cell culture, transient transfection, and treatment with oligonucleotides.** DU145 cells were maintained in DMEM (Life Technologies) with 10% fetal calf serum in PBSTX for 1 h at room temperature, incubated with primary antibody (10 μg/mL of anti-FLAG or 5 μg/mL of anti-nucleolin) overnight (Invitrogen, Inc.) according to the manufacturer’s instructions. For treatment with oligonucleotides, a stock solution (typically 1,000 μmol/L) in water was added directly to the cell culture medium to give the desired final concentration. Except where indicated, the final concentration of oligonucleotide was 10 μmol/L and cells were treated for 24 h before preparation of cell extracts.

**Immunofluorescence.** After 24 h of transfection, cells were plated in eight-well culture slides (Biocoat, Becton Dickinson). After a further 24 h, cells were fixed with 4% paraformaldehyde in PBS for 15 min. After washing with PBSTX (PBS with 0.2% Triton X-100), cells were blocked with 5% goat serum in PBSTX for 1 h at room temperature, incubated with primary antibody (10 μg/mL of anti-FLAG or 5 μg/mL of anti-nucleolin) overnight at 4°C, washed, and then incubated with FITC-conjugated anti-mouse for 1 h at room temperature. Fluorescent images were visualized with a Biorad microscope (Nikon).

**Preparation of protein extracts from DU145 cells.** Nuclear and cytoplasmic extracts were prepared as described previously (15). Fractionation was confirmed by Western blotting for cytoplasmic marker (glyceraldehyde-3-phosphate dehydrogenase) and nuclear marker (lamin). Extracts were either used immediately or stored at −80°C. For whole-cell lysates, cells were mixed with lysis buffer (50 mmol/L Tris-HCl (pH 7.4), with 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, 0.5% IGEPA L CA630) and complete protease inhibitor cocktail (Roche Diagnostics), incubated for 30 min on a shaker at 4°C, and the supernatant was collected after centrifugation.

**Immunoprecipitation assays.** For capture of FLAG-tagged protein, extracts from transiently transfected cells were incubated with 40 μL of washed anti-FLAG beads for 4 h at 4°C. The protein-bound agarose beads were collected and washed five times with buffer. Proteins were eluted with 20 μL of 50 mmol/L Tris-HCl and 150 mmol/L NaCl containing 150 μg/mL of 3× FLAG peptide. For immunoprecipitations of endogenous proteins, 20 μg of extracts from nontransfected DU145 cells were incubated with 2 μg of specific antibody in 50 μL of radioimmuno precipitation assay (RIPA) buffer (PBS, 50 mmol/L Tris-HCl (pH 7.5), 0.5 mol/L NaCl, 0.1 mmol/L EDTA, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/L sodium fluoride, 10 mg/mL phenylmethylsulfonyl fluoride, 2 μmol/L aprotinin, 100 mmol/L sodium orthovanadate) for 1 h at 4°C. MagnaBind goat anti-mouse IgG or goat anti-rabbit IgG beads (250 μL) were added and incubated overnight at 4°C. Beads were captured, washed four times with RIPA buffer, then elu sedimented in 1× SDS-loading buffer [100 mmol/L Tris-HCl (pH 6.8), 200 mmol/mL DTT, 4% SDS, 0.2% bromphenol blue, 20% glycerol] and placed at 95°C for 5 min. Beads were captured and the supernatant containing eluted proteins was removed.

**Electrophoresis and silver staining.** Samples were incubated in 1× SDS-loading buffer at 95°C for 5 min, and separated on 10% polyacrylamide-SDS gels. The silver staining was done as follows: The gel was fixed in 50% methanol, 5% acetic acid, for 30 min with rotation, then sensitized with 0.02% sodium thiosulfate for 2 min. After washing thrice for 5 min with distilled water, the gel was incubated in 0.1% silver nitrate solution for 30 to 60 min at 4°C. After washing twice for 1 min with distilled water, the gel was developed in 0.04% formalin, 2% sodium carbonate until the desired intensity of staining was achieved. Development was terminated with 1% acetic acid solution.

**Protein identification by proteomic analysis by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.** In-gel trypsin digestion was carried out as described (27) with modifications as follows: Protein bands were excised and incubated in 50 mmol/L NH4HCO3/50% acetonitrile at room temperature for 15 min. The gel pieces were allowed to swell by incubating with 20 mmol/L DTT in 0.1 mol/L NH4HCO3 for 45 min at 56°C. After removing this DTT solution, the gel was incubated in 55 mmol/L iodoacetamide in 0.1 mol/L NH4HCO3 for 30 min in the dark. The gel was rinsed with 50 mmol/L NH4HCO3 and incubated in 50 mmol/L NH4HCO3/50% acetonitrile to shrink. After drying in a speedvac, an aliquot of 25 μg/mL sequencing-grade trypsin in 50 mmol/L NH4HCO3 was added. After 45-min incubation on ice, the supernatant was discarded and replaced with 20 μL of 50 mmol/L NH4HCO3. Digestion was done at 37°C overnight and fragmented peptides were extracted from the gel with 5% formic acid/50% acetonitrile. To improve the ionization efficiency of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), ZipTipC18 (Millipore) was used to purify peptides before MS analysis, according to the manufacturer’s manual. The peptides were eluted with 2 μL of 5 mg/mL α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% trifluoroacetic acid and applied directly onto the target and allowed to air dry. Peptide mass fingerprints were obtained using a TOF-Spec 2E MALDI-TOF mass spectrophotometer (Waters). The Mascot program (Matrix Science)5 was used to interpret MS spectra of protein digests.

**Capture of complexes using purified recombinant maltose binding protein–nucleolin fusion proteins.** Plasmids for the expression of maltose binding protein (MBP) fused to nucleolin fragments were a gift from Dr. Nancy MaiZels (University of Washington, Seattle, WA). Recombinant proteins were produced by overexpression in Escherichia coli and purified as described previously (28). Purified MBP-tagged nucleolin fragments (50 pmol) were incubated with 10 μg of whole-cell lysate and 100 μL of amyllose-linked magnetic beads in 500 μL of column buffer [20 mmol/L Tris-HCl (pH 7.4), 0.2 mol/L NaCl, 1 mmol/L EDTA, 10 mg/mL phenylmethylsulfonyl fluoride] for 1 h at 4°C. The beads were precipitated and washed thrice with column buffer, and bound proteins were eluted with 50 μL of SDS loading buffer.

**Small interfering RNA transfection.** One day before transfection, DU145 cells were plated in six-well plates at a density of 2.0 × 10^5 per well in 1.5 mL of DMEM without antibiotics. For each transfection, 200 pmol of nucleolin Stealth RNAi Select (Invitrogen) or control Stealth RNAi Negative Control (Invitrogen) were diluted in 200 μL of Opti-MEM (Invitrogen). In a

5 http://www.matrixscience.com/search_form_select.html
separate tube, 10 μL of LipofectAMINE 2000 (Invitrogen) were diluted in 200 μL Opti-MEM and incubated for 5 min at room temperature. The diluted oligomer and diluted LipofectAMINE 2000 were mixed gently and incubated for 20 min at room temperature. The oligomer-LipofectAMINE 2000 complexes were then added to each well containing cells and medium. Cells were incubated at 37°C in the presence of the transfection solution for 24 h, then the medium was replaced with DME containing 10% FCS.

Western blot analysis. Samples were separated on 8% gels and electroblotted to polyvinylidene difluoride membranes (Millipore). After blocking for 1 h in 5% nonfat dried milk in PBS (0.05% Tween 20 in PBS), the membrane was incubated for 1 h at room temperature or overnight at 4°C with primary antibody. After three washes in PBS, the membrane was incubated with horseradish peroxidase–conjugated goat anti-mouse antibody for 45 min at room temperature, washed thrice in PBS, and detected using enhanced chemiluminescence (GE Healthcare).

Real-time quantitative reverse transcription-PCR, chromatin immunoprecipitation assay, and cell cycle analysis. Total RNA was prepared from untreated and treated DU145 cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The yield and quality of RNA was evaluated by measuring its absorbance at A260/A280 using a NanoDrop spectrophotometer (NanoDrop Technologies). The cDNA was prepared with the Quantitect Reverse Transcription Kit (Qiagen). According to the manufacturer's manual, a total of 1 μg of each sample was included in a 20 μL reaction containing 2 μL of 5× gDNA Wipeout Buffer, 4 μL of 5 × Quantscript RT Buffer, 1 μL of RT Primer Mix, and 1 μL of Quantscript Reverse Transcriptase. One microliter of this mixture was used as template for PCR amplification. Thirty-five PCR cycles were done as follows: 15 s denaturation at 94°C, 15 s annealing at 60°C, and 30 s extension at 72°C. Thermal cycling was done on a DNA Engine Opticon System (MJ Research). Each sample was run in triplicate based on which average copy numbers were calculated. Copy numbers were normalized to β-actin control amplification. Specific primer pairs were used to amplify the following genes: NM23 (+30 to +290, BC000293), ST7 (+548 to +653, BC039954), Cyclin E2 (+135 to +243, BC020729), and β-actin (+140 to +585). Chromatin immunoprecipitation (ChIP) assays were done using the EpiQuick ChIP kit (Epigentek, Inc.) according to the manufacturer's instructions. The promoter sequences amplified have been previously described and were as follows: ST7 −205 to +199 (29) and cyclin E2 −312 to +129 (30). The PCR products were separated on 1% agarose gels and stained with ethidium bromide for visualization. Analysis of cell cycle distribution and differences are indicated as * (P < 0.05) or ** (P < 0.01).

Densitometry and statistical analysis. In some experiments, densitometry was used to measure band intensities by scanning autoradiographic films and using UN-SCAN-IT gel software (Silk Scientific). Band intensities were normalized as indicated in the figure graphic films and using UN-SCAN-IT gel software (Silk Scientific). Densitometry and statistical analysis.

Results

Identification of PRMT5 as a nucleolin-associated protein. We used the pFLAG-nucleolin construct to express FLAG-tagged nucleolin in DU145 prostate cancer cells and confirmed that the epitope-tagged protein had similar distribution to endogenous nucleolin (see Fig. 1A). To isolate nucleolin-associated proteins, we transiently transfected DU145 cells with pFLAG-nucleolin for 72 h, then prepared protein extracts and carried out immunoprecipitation using anti-FLAG antibody. The captured proteins were separated by electrophoresis and visualized by silver staining, followed by tryptic digestion and MALDI-TOF-MS analysis. Using this technique, we were able to identify several bands representing candidate nucleolin-interacting proteins.6 Among these proteins, we focused on validating one particular band whose interaction with nucleolin was altered by AS1411. As shown in Fig. 1B (arrow), this protein was specifically coprecipitated by FLAG-nucleolin and was decreased in immunoprecipitated nuclear extracts from cells treated with AS1411 but unchanged in extracts from cells treated with a control oligonucleotide. This protein was subsequently identified as PRMT5 by mass peptide fingerprinting of trypsin digests using MALDI-TOF-MS (Fig. 1C).

Endogenous nucleolin interacts with PRMT5 and AS1411 alters the localization of nucleolin-associated PRMT5. To evaluate the interaction of endogenous nucleolin with PRMT5 in DU145 cells, we did immunoprecipitation using antinucleolin antibody and probed with anti-PRMT5 antibody. PRMT5 complexes have been reported in both the nuclear and cytoplasmic compartments of the cell (31) and, in this case, PRMT5 was coprecipitated with endogenous nucleolin using either nuclear or cytoplasmic extracts from wild-type DU145 cells (Fig. 1D). The results also clearly showed that nucleolin-associated PRMT5 was decreased in the nucleus of AS1411-treated cells and was increased in the cytoplasm of AS1411-treated cells, compared with untreated cells. The control oligonucleotide had no effect on the localization of nucleolin-associated PRMT5 and there was no apparent change in the levels of nucleolin in extracts from AS1411-treated cells. Neither nucleolin nor PRMT5 could be identified in mock immunoprecipitates using normal mouse IgG or in nucleolin immunoprecipitates without added protein extracts, thus confirming the specificity of the interaction. These findings were highly reproducible and statistically significant (Fig. 1D, bottom).

AS1411 induces nuclear-to-cytoplasmic redistribution of PRMT5 via a nucleolin-mediated mechanism. To further investigate this phenomenon, we analyzed levels of PRMT5 in nuclear and cytoplasmic extracts from AS1411-treated cells by Western blotting. Figure 2A shows that there is a time-dependent decrease in levels of nuclear PRMT5, which is matched closely by a concomitant increase in levels of cytoplasmic PRMT5. The redistribution of PRMT5 was apparent by 4 h after addition of AS1411 and was maximal at 24 h. There was very little change in PRMT5 distribution in cells incubated with the control oligonucleotide. Using similar techniques, we determined that the redistribution of PRMT5 also depends on the dose of AS1411 (see Fig. 2B). The degree of PRMT5 relocation seen in these Western blots analyzing total PRMT5 was comparable with that seen in the previous experiments, which examined nucleolin-associated PRMT5 (compare Fig. 1D with the 24 h time point in Fig. 2A), suggesting that it is the nucleolin-associated PRMT5 that is altered by AS1411. To test our hypothesis that the AS1411-induced relocation of PRMT5 is mediated by nucleolin, we next examined cells that had been transfected with a small interfering RNA (siRNA) that specifically depletes nucleolin (Fig. 2C). In these cells, the AS1411-induced changes in PRMT5 were almost completely abrogated, whereas the effect of AS1411 on PRMT5 persisted in cells treated with a control siRNA.

AS1411 changes the distribution of sDMA—a modification that is catalyzed by PRMT5. Protein arginine methyltransferases are classified as type II enzymes, which catalyze the symmetrical

6 Y. Teng et al., in preparation.
dimethylation of target arginines, or type I arginine methyltransferases, which catalyze asymmetrical dimethylation (31–36). PRMT5 is thought to be the major type II enzyme that is responsible for sDMA modifications in most cell types. Therefore, to investigate the effect of AS1411 on the activity of PRMT5, we analyzed levels of sDMA in extracts from AS1411-treated and control-treated DU145 cells by Western blotting using an antibody named SYM10, which specifically recognizes sDMA (36). As a control for specificity, we also used an antibody named ASYM24, which recognizes aDMA-containing proteins (36). Extracts from the nucleus and cytoplasm of AS1411-treated, control-treated, and untreated DU145 cells were analyzed by Western blotting using either SYM10 or ASYM24 and the recognition patterns of the two antibodies were found to be quite distinct, confirming their specificity (see Fig. 3A). Moreover, the results shown in Fig. 3A indicate that treatment with AS1411 results in a specific decrease...
in the intensity of bands recognized by SYM10 in the nuclear extracts, with a corresponding increase in the intensity of SYM10-immunoreactive bands in the cytoplasmic extracts. In contrast, there were no changes in the bands detected by ASYM24 in extracts from AS1411-treated cells. Because the sDMA modifications recognized by SYM10 are presumably an indicator of PRMT5 activity, these data are consistent with the observed nuclear-to-cytoplasmic redistribution of the enzyme.

The nucleolin complex contains sDMA modifications and is a likely substrate for PRMT5. Because nucleolin is associated with PRMT5 and contains an arginine-rich region (the "RGG domain"), which is a common target for PRMTs (31–36), we wondered if nucleolin itself could be a substrate for PRMT5. To test this idea, we immunoprecipitated with the SYM10 antibody and then Western blotted using antinucleolin antibody. As indicated in Fig. 3B, the anti-sDMA antibody was able to specifically precipitate nucleolin. Furthermore, there was a nuclear-to-cytoplasmic shift in levels of sDMA-precipitated nucleolin in AS1411-treated cells, similar to that seen for PRMT5. This was in contrast to the levels of total nucleolin, for which there was no obvious change (see the nucleolin blot in Fig. 1D), suggesting that sDMA-modified nucleolin may represent only a small proportion of the total nucleolin pool.

Figure 2. AS1411 alters the subcellular distribution of PRMT5 in a time-dependent, dose-dependent, and nucleolin-dependent manner. Nuclear and cytoplasmic extracts were prepared from DU145 cells that had been treated with either AS1411 (A) or control oligonucleotide (C). Extracts were analyzed by Western blotting for PRMT5, then for β-actin (a control for equal loading). A, cells were incubated with 10 μmol/L of AS1411 for the times indicated. B, cells were incubated for 24 h with oligonucleotide at the concentrations indicated. Data points, mean of three independent experiments; bars, SE. C, cells were transfected with either nucleolin siRNA or control siRNA for 48 h before treatment with 10 μmol/L oligonucleotide and then analyzed by Western blotting after a further 24 h. Data from a single representative experiment. All panels show representative Western blots and quantification of the results using densitometry. The intensity of the PRMT5 band in each sample was normalized to the corresponding β-actin band and expressed relative to the untreated sample.
Therefore, we postulated that only a small fraction of the total nucleolin is associated with PRMT5, whereas most of the PRMT5 exists in a complex with nucleolin. This would explain why a large proportion of the total PRMT5 is translocated in AS1411-treated cells by 24 h (Fig. 2A), whereas levels of total nucleolin are virtually unchanged by AS1411 (Fig. 1D). To test this theory, we estimated the fraction of total nucleolin that was associated with PRMT5, and vice versa, by analyzing the amount of protein immunoprecipitated with and after immunoprecipitation with nucleolin antibody. The results (Fig. 3C) indicate that a large proportion (>78%) of the total PRMT5 coprecipitates with nucleolin. We were not able to directly assess PRMT5-associated nucleolin in a similar way because the PRMT5 antibody was not efficient for immunoprecipitation. However, by immunoprecipitating with sDMA antibody, we confirmed that only a small fraction (<6%) of nucleolin was sDMA-modified, which presumably reflects the amount of nucleolin that is bound to PRMT5 (Fig. 3D).

**Association with PRMT5 is mediated by the RGG domain of nucleolin.** To further investigate which domain of nucleolin is responsible for the interaction with PRMT5, binding assays were done using several recombinant nucleolin polypeptides (see Fig. 4A). These recombinant proteins contained the first two RNA binding domains (RBD1,2) and/or the arginine-glycine repeat (RGG) domain fused at their NH2-terminal end to a MBP tag. Only these domains were investigated because the NH2-terminal domain of nucleolin cannot be expressed in E. coli and the RBD3,4 domain is subject to partial proteolysis,7 probably by autodegradation (10).

7 P.J. Bates, unpublished observation.
After in vitro incubation of purified MBP-fusion proteins with DU145 cell lysates, bound proteins were recovered using maltose affinity gel beads. As shown in Fig. 4B, no PRMT5 was recovered in the precipitate when the recombinant protein contained the MBP tag alone or MBP-tagged RNA binding domains (RBD1,2). In contrast, when MBP was fused to either RGG or RBD1,2-RGG, PRMT5 was precipitated. These results indicate that the RGG COOH-terminal domain of nucleolin is the minimal domain required for interaction of nucleolin with PRMT5 and that RBD1,2 can promote the RGG-PRMT5 interaction. When the same samples were Western blotted using the SYM10 antibody, there was also strong staining of a band corresponding to the MBP-RBD1,2-RGG fragment, confirming our previous postulate that nucleolin is a substrate for sDMA modification by PRMT5.

**AS1411 causes up-regulation of some PRMT5 target genes, including tumor-suppressor ST7 and cyclin E2.** Previous work by Pal et al. (29) has shown that recruitment of PRMT5 to the promoter regions of tumor-suppressor genes, ST7 and NM23, leads to transcriptional repression of these genes due to sDMA modification at R8 of histone H3. These two genes, and the cyclin E2 gene, were up-regulated in cells when antisense RNA was used to reduce levels of PRMT5 (29). Based on our observations that levels of PRMT5 are reduced in the nuclei of AS1411-treated cells and because PRMT5 acts as a transcriptional repressor, we predicted that PRMT5 target genes would be up-regulated in AS1411-treated cells. To test this theory, we analyzed expression levels of selected PRMT5 target genes by real-time reverse transcription-PCR (RT-PCR) using RNA from untreated, AS1411-treated, or control-treated DU145 cells. As shown in Fig. 5A, AS1411-treated DU145 cells exhibited a 3.9-fold and 2.2-fold higher expression of cyclin E2 and ST7 mRNA, respectively, compared with untreated cells. These values are comparable with the changes observed in the previous study where antisense RNA was used to deplete PRMT5 in NIH-3T3 cells (29). However, in contrast to the previous study, expression of the NM23 gene was not significantly changed in AS1411-treated DU145 cells, suggesting that regulation by PRMT5 may be cell type dependent. To confirm that the observed changes in ST7 and cyclin E2 expression were due to decreased binding of PRMT5 to the promoter regions of those genes, we next did ChIP experiments. These showed that the levels of PRMT5 associated with the cyclin E2 and ST7 promoters were significantly decreased in cells treated with AS1411 (see Fig. 5B), whereas the control oligonucleotide had no effect on PRMT5 (Fig. 5B) and AS1411 did not affect the positive control ChIP (data not shown). Because cyclin E2 is a cell cycle regulator that accelerates G1-S, we also examined if there was a perturbation of the cell cycle in AS1411-treated cells. We found that there was a small but significant increase in the proportion of cells that were in S phase and a reduction in G2-M (Fig. 5C).

**Discussion**

Protein arginine methylation (R-methylation) is a posttranslational modification that results in the addition of one or two methyl groups to the guanidino nitrogen atoms of arginine. There are at least nine human PRMTs responsible for arginine methylation and they have been classified into two major classes: Type I enzymes promote the formation of asymmetrical $\alpha-N^6, N^\epsilon$-dimethyl arginines (aDMA) and type II enzymes catalyze the formation of symmetrical $\alpha-N^6, N^\epsilon$-dimethyl arginines (sDMA), whereas $\alpha-N^\epsilon$-monomethylarginine is thought to be an intermediate formed by both types (31–36). Consensus sequences for various PRMTs have not been defined, although most R-methylated proteins identified to date are modified at glycine/arginine-rich repeats. The biological function of arginine methylation is also not yet fully understood, but there is credible evidence for roles in transcriptional regulation, RNA processing, signal transduction, DNA repair, and subcellular protein transport. These roles have been detailed recently in several excellent reviews about arginine methylation (31–36).

In this study, we have identified a specific interaction between nucleolin and the major type II arginine methyltransferase, PRMT5. This seems to be a novel finding because, although an earlier proteomics analysis identified PRMT5 (also known as JBP1 and SKB1) as a protein that coprecipitated with nucleolin, the PRMT5-nucleolin interaction was previously dismissed as nonspecific (37). In contrast, our current work clearly shows that the PRMT5-nucleolin interaction is specific and occurs in cultured DU145 prostate cancer cells. Our data also indicate that PRMT5-associated...
nucleolin contains sDMA, which strongly suggests that nucleolin is a substrate for modification by PRMT5. Interestingly, the RGG domain of nucleolin has been previously shown to be a substrate for asymmetrical dimethylation by the type I enzyme, PRMT1 (38). However, it is not unprecedented for a protein to be a target for both types of PRMTs and there is mounting evidence that there can be competition between type I and type II enzymes for the same arginines, with aDMA or sDMA modifications having opposing effects (reviewed in ref. 31). An example is the R3 residue of histone H4, which can be modified by PRMT1, leading to transcriptional activation, or by PRMT5, which results in transcriptional repression. Another protein that can contain both aDMA and sDMA modifications is the sliceosomal protein, SmB, and, in this case, the type of arginine methylation was related to the nuclear or cytoplasmic localization of the protein. In terms of cell biology, the significance of nucleolin methylation is not yet known. Its role in nucleolar localization and nucleic acid binding has been studied, but methylation of the RGG domain was found to be nonessential for
these functions (39, 40). It may be relevant that there is substantial overlap between the biological functions of nucleolin and PRMT5; for instance, both have been implicated in transcriptional regulation, chromatin remodeling, spermatocyte maturation, RNA processing, complex formation with SMN1, and mediating nuclear/cyttoplasmic localization (10–12, 31–36, 41–47). It is also tempting to speculate that the apparently inconsistent roles (sometimes activating, sometimes repressing) of nucleolin in transcriptional regulation (reviewed in refs. 10–12) could be explained in terms of whether it is associated with (or methylated by) PRMT1 or PRMT5. In short, further research will be required to fully elucidate the relationships involved, but the results presented herein clearly point to an intriguing link between nucleolin biology and arginine methylation, especially with regard to transcriptional regulation and nuclear-cyttoplasmic shuttling.

Another major result of this study is the discovery that the nucleolin-targeted aptamer, AS1411, can alter the cellular localization and activity of PRMT5. This has a number of important implications for the mechanism of this novel anticancer agent. The role of PRMT5 in cancer biology has not been widely studied, but there is some evidence that increased PRMT5 levels (particularly, nuclear PRMT5) are associated with malignant transformation. A recent report identified PRMT5 (alternatively called SSB1) as a gene that was specifically overexpressed, at both the mRNA and protein levels, in gastric cancers compared with normal gastric tissues (48). Immunohistochemistry studies comparing normal gastric tissue with moderately and poorly differentiated carcinomas found that, not only was there an increase in the overall levels of PRMT5, but that there was a shift from cytoplasmic to nuclear staining as the degree of malignancy increased (48). Other evidence for the significance of PRMT5 in cancer comes from Pal et al. (29). These authors have shown that stable expression of FLAG-tagged PRMT5 can transform NIH-3T3 cells, such that they proliferated faster than wild-type cells and could grow in an anchorage-independent manner. Conversely, cells that were stably transfected with antisense PRMT5 cells grew considerably slower than wild-type cells. To investigate the mechanism of this effect, these researchers carried out microarray studies to compare gene expression in cells expressing antisense PRMT5 (which had PRMT5 mRNA levels reduced by >90%) with wild-type cells. Two tumor suppressors, ST7 and NM23, were found to be up-regulated in the antisense cell line and were subsequently identified as direct targets of PRMT5-mediated transcriptional repression. The cell cycle regulator, cyclin E2, was also induced in the antisense PRMT5 cells, although it was not determined if this was a direct effect. We have shown here that treatment of DU145 prostate cancer cells with AS1411 reduces the levels of PRMT5 in the nucleus, thereby causing an increase in ST7 and cyclin E2 expression due to a decrease in the amount of PRMT5 that is associated with their promoter regions. Another recent report described how a nuclear- to-cytoplasmic shift of a PRMT5 repressor complex resulted in up-regulation of target gene expression (49). We predict that AS1411-induced derepression of ST7 and cyclin E2 genes, and possibly other PRMT5 target genes that have not been identified here, may contribute to the biological activity of AS1411. The human ST7 gene was first recognized as a candidate tumor suppressor based on its chromosomal location (7q31.1) at a site of frequent loss of heterozygosity and its reduced expression in some types of cancer (50). Multiple studies failed to find tumor-associated mutations in the ST7 gene (reviewed in ref. 50), suggesting that inactivation is due to epigenetic silencing at the level of chromatin organization, probably mediated by PRMT5 (29, 51). More importantly, the role of ST7 as a tumor suppressor has been shown at the functional level. Studies have shown that ectopic expression of the ST7 gene in human breast or prostate cancer cells resulted in reduced anchorage-independent growth and suppression of tumorigenicity in immunodeficient mice (50). Thus, AS1411-induced up-regulation of ST7 could evidently contribute to its anticancer activity. The possible role of cyclin E2 up-regulation in the mechanism of AS1411 is less obvious because this gene is a cell cycle regulator that controls the G1-S transition and is generally considered to be growth promoting. Nonetheless, it is worth noting that the antisense PRMT5 cells grew slower than wild-type cells, even while cyclin E2 levels were enhanced (29). Also, cancer cells treated with AS1411 are accumulated in S phase (Fig. 5C), consistent with previous reports that GRO29A (a longer version of AS1411) induces cell cycle arrest in S phase (4).

Our results have additional broader implications in terms of the mechanism of AS1411 activity. Previous work has indicated that GROs bind directly and specifically to nucleolin protein (3), and that antiproliferative activity across a series of GROs is correlated with their nucleolin binding affinity (3, 5, 6). These data support our assertion that GROs, including AS1411, work as nucleolin-targeted aptamers. On the other hand, no obvious changes in the levels or localization of nucleolin are observed in cancer cells treated with AS1411 or other active GROs. Therefore, we hypothesized that AS1411 modulates the activity of nucleolin by altering its posttranslational modifications and/or affecting its interactions with other proteins. Our previous research (7) has identified NEMO/IKKγ, which is a critical factor in nuclear factor-κB (NF-κB) activation, as a protein that coprecipitated with AS1411 and nucleolin. In that study, we found that the presence of AS1411 increased the association of NEMO with nucleolin in the cytoplasm, resulting in abrogation of NF-κB signaling (7). In this article, we have described how AS1411 affects both a posttranslational modification (symmetrical arginine dimethylation) of nucleolin and the localization of a specific nucleolin complex (with PRMT5). Interestingly, these changes were linked to nuclear-to-cytoplasmic redistribution of the nucleolin-PRMT5 complex, suggesting that the shuttling function of nucleolin may be affected by AS1411. Moreover, only a small proportion of cellular nucleolin, consisting of the sDMA-modified protein, was affected by AS1411, which explains why there is no obvious change in total nucleolin levels in cells treated with AS1411. Taken together, our results support the hypothesis that aptamer-induced perturbations of nucleolin (or a subset of nucleolin complexes) are responsible for the biological effects of AS1411. Therefore, in addition to being a promising therapeutic agent, AS1411 may be a useful tool for investigating the regulation and functions of this extraordinary protein. This may ultimately lead to important insights into tumor biology, because there is mounting evidence that overexpression of nucleolin can contribute to cancer development and progression (11).

Acknowledgments

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Conflict of interest: P.J. Bates owns significant stock in Antisoma PLC (London, England), the company that is developing AS1411.

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References


AS1411 Alters the Localization of a Complex Containing Protein Arginine Methyltransferase 5 and Nucleolin

Yun Teng, Allicia C. Girvan, Lavona K. Casson, et al.


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