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Nucleolin overexpression confers increased sensitivity to the anti-nucleolin aptamer, AS1411

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Abstract

AS1411 is an antiproliferative DNA aptamer which binds the ubiquitous protein, nucleolin. In this study we show that constitutive overexpression of nucleolin confers increased sensitivity to the growth inhibitory effects of AS1411. HeLa cells overexpressing nucleolin have an increased growth rate and invasiveness relative to control cells. Nucleolin overexpressing cells demonstrate increased growth inhibition in response to the AS1411 treatment which correlates with increased apoptosis and cell cycle arrest, when compared to non-transfected cells. AS1411 induces nucleolin expression at the RNA and protein level in HeLa cells, suggesting a feedback loop with important implications for the clinical use of AS1411.

Keywords

Nucleolin; AS1411; G-quadruplex; cervical cancer

Introduction

AS1411 is a 26bp unmodified phosphodiester oligonucleotide which inhibits the growth of transformed, but not normal cells.[1–3] AS1411 forms a highly stable four-stranded dimer, stabilized by G-quartet formation, which is resistant to exonuclease and endonuclease digestion [4]. Although its exact mechanism of action is not known, AS1411 has growth inhibitory activity over a wide spectrum of tumor cell lines. In a Phase I Clinical Trial, 31 patients with advanced solid tumors refractory to conventional/standard treatment were treated with a four or seven day continuous infusion of AS1411 at escalating dosages. Several patients in this clinical trial demonstrated clinical responses in the absence of significant toxicity.[2] Based on this trial, it is clear that AS1411 is well tolerated with no serious toxicity at a dosage of 1–40 mg/kg/day. AS1411 has subsequently been tested in Phase II clinical trials in acute myelogenous leukemia and renal cell carcinoma sponsored by Antisoma, Inc.[2, 3] AS1411 and many other G-quadruplex forming oligonucleotides have been shown to bind with high affinity to nucleolin [1, 5] a ubiquitous protein that is particularly abundant at the surface of cancer cells. The ability of AS1411 to bind to nucleolin and subsequently enter the cytosol has been utilized by several groups to deliver drugs and/or dyes to cancer cells [6, 7].

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Nucleolin is a multifunctional nucleolar phosphoprotein which is highly expressed in exponentially growing eukaryotic cells.[8] It is the most abundant protein in the dense fibrillar core and granular regions of the nucleolus.[9] Nucleolin is a RNA-binding protein (RBP) which plays important roles in the regulation of cell proliferation and growth, cytokinesis, replication, embryogenesis and nucleogenesis.[8, 10] Its diverse molecular functions include roles in ribosomal biosynthesis, RNA binding, DNA and RNA helicase activity and DNA dependent ATPase activity among others.[10] Nucleolin function is regulated at different levels by phosphorylation (by CKII, CDC2, PKC- ϵ , cyclic AMP-dependent protein kinase and ecto-protein kinase)[8, 11–13], ADP-ribosylation, methylation[10] and SUMOylation [14] which confirms its central role in regulating cell growth. Dysregulation of nucleolin has been shown to occur in many pathologic conditions, including viral infection, autoimmune diseases, Alzheimer's disease or Parkinson disease and frequently occurs in cancer [15–19]

In addition to its nucleolar localization, nucleolin is also present in the cytoplasm and on the cell surface where it may act as a surface receptor [20], a role that appears to be important in AS1411 internalization and transport. One of its diverse roles is to serve as a shuttling protein between the cytoplasm and nucleus [8, 21, 22], providing a mechanism for extracellular regulation of nuclear events.[8] In addition, nucleolin has been shown to promote the maturation of a specific set of miRNAs implicated in the pathogenesis of several human cancers, including *miR-21*, *miR-103* and *miR-222*. [23] Nucleolin is known to be a significant component of AgNORs whose over-expression has been shown to correlate with poorer clinical outcomes and shorter survival in studies with several tumor types.[24–38]

In non-dividing cells, auto-degradation of nucleolin leads to a preponderance of truncated forms of various molecular sizes.[39, 40] In actively dividing cells, the intact form of the protein predominates and represents as much as 5% of nucleolar protein. Changes in the level of expression of a multifunctional protein such as nucleolin would likely have significant repercussions on the phenotype of the affected cells. Its over-expression has been reported in the context of experimental hepatic carcinogenesis[35] and, in one report, its downregulation by antisense oligonucleotides inhibited cellular proliferation in HPV 18 (Human Papilloma Virus) positive cervical carcinoma cell lines.[38] We have shown that AS1411 binds cell surface nucleolin and inhibits proliferation of transformed cells.[1, 3] Recent work by Croce's group has shown that both AS1411 and anti-nucleolin antibodies inhibit the proliferation of a variety of tumor cell types.[23, 41]

In the work reported here, we have addressed the effect of increased nucleolin expression on the ability of AS1411 to inhibit the proliferation of HeLa cells. We characterized the effect of constitutive overexpression nucleolin on HeLa cell growth rate, invasiveness, response to anti-tumor agents. Most importantly, we demonstrate that constitutive overexpression of nucleolin markedly increases cell sensitivity to the nucleolin-binding DNA aptamer, AS1411. Paradoxically, we also demonstrate that AS1411 induces higher level of expression of nucleolin in these cells that will eventually precipitate their death.

Materials and Methods

Cell Culture

HeLa (cervical cancer) cells were obtained from American Type Culture Collection (ATCC, Manassas, VA), cultured and maintained in Dulbecco's Modified Minimal Essential Medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS) and Penicillin G (100 units)-Streptomycin (100 µg) (all from HyClone, Utah). Cultures were grown in humidified incubators at 37°C and 5% CO₂.

Cloning and Transfection

Nucleolin cDNA was expressed in the pCMVscript expression vector. Full length human nucleolin cDNA was first excised from pBlueScript using the restriction enzymes EcoRI and XhoI and ligated into the MCS of pFastBac. Next, it was excised from pFastBac with BamHI and XhoI and ligated at the same restriction sites in the sense direction into the MCS of pCMVscript. The resulting constructs were confirmed by restriction analysis and DNA sequencing to contain full length nucleolin cDNA in sense orientation. Wild type HeLa cells were plated at 1×10^6 cells per 100mm culture dish overnight. The following day each nucleolin construct (10µg) was added to DOTAP:DOPE [cationic lipid DOTAP (*N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium chloride) and neutral lipid DOPE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine) Sigma-Aldrich, MO] (1:4 ratio) and incubated at room temperature for 15min, then added to the cells for 24h. Fresh media was added the next day. Twenty-four hours later, cells were split 1:10. Selection was started 24h later with 800µg/ml of Geneticin (Invitrogen-Life Technologies Corporation, NY). Geneticin was reduced by half for several weeks until 200µg/ml was obtained. Single clones were isolated and plated in 48 well culture plates and expanded sequentially to 25cm² flasks. The clone S7 was selected for further analysis. For some experiments, pooled colonies were cultured directly on a large scale (SP). Transfected cells were maintained in complete DMEM plus 100µg/ml of Geneticin.

Western blot

Whole cell lysates were obtained by lysing cells in M-PER mammalian protein extraction reagent (Thermo Fisher Scientific-Pierce #78501) with 1x protease inhibitor (Roche Diagnostics, IN #1836153). Proteins were resolved on an 8% SDS polyacrylamide gel and transferred to PVDF membranes. PVDF membranes were blocked at room temperature with 5% non-fat dry milk in PBST (PBS plus 0.5% Tween-20) for 1h, washed 5 min (3x) with 0.5% PBST. After washing, membranes were incubated with a 1:1000 dilution of a mouse anti-nucleolin antibody (C23 clone D-6, Santa Cruz Biotechnology, TX) for 1h at room temperature, washed as described above and further incubated with a 1:2000 dilution of horseradish peroxidase-conjugated goat anti-mouse secondary antibody (diluted in PBST) for 1h at room temperature. After three washes at room temperature, proteins were detected using enhanced chemiluminescence (Amersham Pharmacia) and visualized using Kodak film.

MTT Proliferation Assay

The tetrazolium dye (3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide, Sigma-Aldrich, MO) MTT was used to assay proliferation. HeLa, HeLa-S7 and HeLa-SP cells were plated at 2.5×10^3 cells per well in triplicates in 96-well plates and measured for cell growth at 24h and 48h. In some experiments cells were allowed to grow for 24h before being treated with Cisplatin (Sigma-Aldrich) at different doses (1 μ M up-to 50 μ M), with Camptothecin (Sigma-Aldrich) at dose between 10nM and 10 μ M or with AS1411 (Oligos Etc., OR) at dose between 1 μ M and 10 μ M as shown in the results section for 3 days. At the end of the culture time, the dye was added and incubated for 4h at 37°C with 5% CO₂. Following incubation, lysis buffer (10%SDS in 0.01N HCl) was added and samples incubated overnight absorbance was measured on a plate reader at 570 nm. After normalization to the blank (media only) data were normalized to the untreated and expressed as percent of the control, averages for 3 separate experiments $\pm$ SEM are shown.

In vitro cell invasion assay

Invasiveness through a Matrigel® coated membrane (Becton-Dickison, NJ) was assayed as previously described.[42] Briefly, Matrigel® inserts were rehydrated with 37°C medium for 2h. For each Matrigel® insert, an eight micron control insert was prepared. Fetal bovine serum was added as chemoattractant to each well prior to adding inserts. Control and Matrigel® inserts were put in individual wells and 0.5 ml of cell suspension (HeLa-CMV, HeLa-S7 and HeLa-SP cells at 1×10^3 /ml in DMEM containing 0.1% BSA) was added in each insert. Plates were incubated for 24h at 37°C and 5% CO₂ in a humidified incubator. Following incubation, non-invading cells were removed from the upper surface of the membrane by scrubbing with a cotton tipped swab. Inserts were stained with the Hematoxylin/ Eosin Diff-Quick stain (Baxter Diagnostics, IL #B4132-1) briefly: the inserts were transferred through solutions of fixative and hematoxylin and eosin for 30 seconds each and air-dried for 1h. Membranes were removed with a #11 blade and photographed at 100X magnification. For quantification, stained cells were removed from the membrane with 10% acetic acid and read at 570 nm in plate reader.

Flow-cytometry analysis

HeLa, HeLa-S7, HeLa-SP cells were seeded at 6×10^3 /ml in 100mm culture Petri dish and allowed to attach for 24h then treated with 10 μ M AS1411 for 3 days. At the end of the culture, cells were collected using Accutase® (StemPro®Accutase®, Gibco/Life Technologies) washed in PBS, counted in Trypan Blue and divided in 2 aliquots for Western Blot and FACS analysis.

Apoptosis assay

Cells collected were re-suspended in 3ml binding buffer and washed one more time. Cells were then re-suspended at 1×10^6 /tube in 100 μ l binding buffer and 10 μ l of AnnexinV-FITC (BD-Pharmingen, CA) and 5 μ l on 7AAD (eBioscience, CA) were added in each tube and incubated on ice for 20min. After adding 400 μ l of PBS to the tubes samples were run on FACSCalibur (Becton-Dickinson, NJ), analysis was performed using FSC Express4 software

(DeNovo software, CA). Data are expressed as the average of percentage of live and apoptotic cells ($\pm$ SEM) for three separate experiments.

Cell cycle assay

Cells collected were re-suspended in 1ml ice-cold 70% ethanol and stored at -20°C until all samples for three experiments were collected. Cells were then washed in PBS and re-suspended in 500 μl of PBS to which 5 μl of 7AAD were added. After 20 min incubation, cells were run on FACSCalibur and analyzed using the Multicycle program from FSC Express4 software. Data represent the average ($\pm$ SEM) of the percentage of cells in a given sub-population for three separate experiments.

AS1411 cellular up-take

HeLa, HeLa-pCMV, HeLa-S7 and HeLa-SP were cultured in the presence of AS1411-FAM labeled at 5 μM for 3 days. At the end of the culture, cells were collected and fixed in 4% formaldehyde and analyzed by Flow-cytometry on FACSCalibur using FSC Express 4 software.

Nucleolin expression

HeLa, HeLa-pCMV, HeLa-S7 and HeLa-SP were cultured in the presence of AS1411-FAM labeled at 5 μM for 3 days. At the end of the culture, cells were collected and fixed in 4% formaldehyde, permeabilized in PBS/0.1% Triton X/1% BSA/5%FBS and labeled with antibody against Nucleolin (C23-D6, Mouse, Santa Cruz at 1/50 dil) overnight, then incubated with a secondary antibody Goat anti mouse-AF680 for 1h. After washing the cells in PBS, the samples were analyzed by Flow-cytometry on FACSCalibur using FSC Express 4 software.

Immuno-histochemistry

HeLa, HeLa-S7, HeLa-SP cells were seeded at $5 \times 10^3/\text{ml}$ in 8 chamber slides and allowed to attach for 24h then treated with 5 μM AS1411- Cy5 labeled for 3 days. At the end of the culture, cells were washed with PBS and fixed with 4% formaldehyde. The cells were permeabilized and blocked in PBS/0.1% Triton X/1% BSA/5%FBS then labeled with antibody against Nucleolin (C23-D6, Mouse, Santa Cruz at 1/50 dil) overnight at 4°C in the dark. After 3×5 min washes in PBS, cells were incubated for 1h with Goat-anti-mouse-AF488 (1/200 dil, Molecular Probes) at 37°C , washed in PBS and incubated 5min with DAPI (4',6-diamidino-2-phenylindole, Sigma Aldrich) then washed again. The slides were then mounted in Fluoromount G (Southern biotechnology, AL). Photomicrographs were taken using Nikon T2000 and image analysis performed using MetaMorph software (Molecular Devices, CA).

Statistical analysis

All data are expressed as mean $\pm$ SEM between untreated versus treated for at least three separate experiments and assessed using unpaired two-tailed Student T-test and one-way ANOVA. Data were considered significant for p -value < 0.05 .

Results

Constitutive overexpression of nucleolin increases cell proliferation

The effect of constitutive overexpression on nucleolin in HeLa (cervical cancer) cells was characterized by transfection of cultured cells with nucleolin sense expression vector. Nucleolin expression was verified by Western Blot analysis of protein extracted from control HeLa cells (non-transfected cells); HeLa transfected with the empty vector (pCMV); and HeLa cells from selected colonies of sense as well as pooled selected sense transfected cells (SP). The increased level of nucleolin expression was confirmed in clones isolated from HeLa cells stably transfected with sense nucleolin constructs such as HeLa-S7 and in HeLa-SP (Fig. 1A), with a 2 to 2.5 fold increase in the band corresponding to the full length nucleolin the clones containing the sense constructs (HeLa-S7) compared to control cells (Fig. 1A bottom). In addition, the total number and intensity of the lower bands, which corresponds to self-cleavage of the protein, was also higher for HeLa-S7 compared to the control that correlates with highly proliferating cells [39]. Nucleolin mRNA also increased as confirmed by RT-qPCR (Fig. 1B), showing a 6 fold increase of nucleolin gene expression in HeLa-S7 and 2 fold increase in the pooled sense clones compared to the empty vector transformed HeLa cells. The difference in mRNA expression between HeLa-S7 and HeLa-SP could be due to the irregularity of the transfection of the clones that were pooled.

In order to characterize the phenotypic effects of nucleolin overexpression, we compared the growth rate of the nucleolin transfected HeLa-S7 and HeLa-SP cells to control HeLa cells incubated for 24 and 48 hours. Cell proliferation was evaluated using the MTT assay. The HeLa clone expressing the sense vector (S7), shows a significant increase in cell growth and the pooled transfected HeLa cells demonstrated modestly increased cell numbers at 24 hours, compared with the controls (Fig. 1C). While transfected cells HeLa-S7 appeared to grew more rapidly initially (difference between HeLa-S7 and control HeLa cells was larger at 24h), they slowly lost this growth advantage as a function of the time in culture. This suggests that constitutive overexpression of nucleolin may influence cell proliferation only at times when the cells are growing at low density. This is in good agreement with the previous literature which has demonstrated varying amounts of growth stimulation by nucleolin overexpression[43].

Nucleolin expression increases in vitro invasiveness

It has been suggested that overexpression of nucleolin correlates with a more aggressive malignant phenotype [16, 23, 44]. The ability of tumor cells to metastasize and invade other tissues is one characteristic of aggressive cancer and is generally associated with poor prognosis. Therefore, we evaluated whether increased nucleolin expression altered HeLa cells mobility/invasion abilities. Stably transfected cells which overexpressed nucleolin (S7 and SP) were compared to HeLa-control vector transfected cells (pCMV) in Boyden chamber migration assay. HeLa-S7 and HeLa-SP cells which constitutively overexpressed nucleolin showed increased migration after 44h compared to the control HeLa-pCMV cells (HeLa cells are naturally highly mobile) (Figs. 2A and 2C). In addition, nucleolin overexpression induced a change in cell morphology as evidenced by the smaller size and elongated shape of HeLa-S7 and HeLa-SP cells that is compatible with migrating

phenotype. Accordingly, invasiveness of the sense nucleolin over-expressing clones exceeded that of the control HeLa-pCMV cells by greater than ten-fold, as depicted at 14h and 44h (Fig. 2B and 2D). Although the mechanism by which nucleolin overexpression stimulates invasion is not clear, previous work has demonstrated that nucleolin overexpression stimulates expression of the matrix-metalloproteinase-2 (MMP-2) gene, presumably by enhancing MMP-2 mRNA stability [45]. This data demonstrated that overexpression of nucleolin increases HeLa cells ability to invade other tissues in a manner consistent with the increased aggressiveness of nucleolin overexpressing cells.

HeLa cells overexpressing nucleolin are slightly more sensitive to Cisplatin and Camptothecin

Since nucleolin overexpression has been associated with more aggressive behavior of human tumors [26, 46], it was important to characterize the effect of constitutive nucleolin overexpression on sensitivity to chemotherapeutic agents. Therefore, HeLa, HeLa-pCMV, HeLa-S7 and HeLa-SP were treated with Cisplatin (Fig. 3A) or Camptothecin (Fig. 3B) at increasing doses for 3 days and cell proliferation was evaluated by MTT assay. There was an increase in Cisplatin-induced growth inhibition in HeLa-S7 compared to the non-transfected control cells at both low and high doses of the drug (Fig. 3A). However, HeLa-pCMV had also increased sensitivity at high dose compared to HeLa non-transfected cells suggesting a possible additional effect due to the transfection. The nucleolin overexpressing cells (HeLa-S7 and HeLa-SP) were also slightly more sensitive to Camptothecin than were non-transfected HeLa cells (~20% inhibition) at 10nM but that effect was lost at higher doses (Fig 3B). The growth inhibition induced by Camptothecin at high dose (10 μ M) was comparable in all clones and reached 70–75%. This observation most likely reflects the increased proliferative rate of the nucleolin overexpressing cells. Altogether the data show that nucleolin overexpression in HeLa did not significantly increase their response to chemotherapeutic agents. It is also clear that both the non-transfected HeLa cells and the transfected clones have a high sensitivity to 10 μ M Camptothecin.

Nucleolin overexpression sensitizes HeLa cells to AS1411

The important role of nucleolin in tumor cell biology and the fact that it binds the anti-proliferative DNA aptamer, AS1411 [1, 2] raises the question of whether sensitivity to AS1411 may be proportional to nucleolin expression. We hypothesized that nucleolin overexpression would confer increased sensitivity to AS1411. HeLa, HeLa-S7 and HeLa-SP cells were cultured in the presence or absence of AS1411 (1–10 μ M) for 3 days and growth inhibition was measured by the MTT assay. As shown in Fig. 4, the nucleolin transfected HeLa-S7 and HeLa-SP cells were significantly more sensitive to the growth inhibitory effects of AS1411 than were control HeLa cells. This effect was especially marked at the low doses (3 and 5 μ M).

To further characterize the relationship between nucleolin expression and AS1411 sensitivity, cells were treated for 3 days, collected and assayed using Trypan Blue exclusion to determine the total number of cells and the proportion of live/dead cells recovered after the culture. As expected, and even at this earlier time point, AS1411 treatment reduced cell growth significantly in all the treatment groups (Fig. 5A) and correlate with the MTT data.

Furthermore, nucleolin overexpression significantly increased the sensitivity to AS1411 in HeLa-S7 compared to control HeLa cells (Fig. 5A bracket). The decrease in cell proliferation observed in presence of AS1411 in cells overexpressing nucleolin was associated with a significant increase in the frequency of dead cells when HeLa-S7 cells were compared to control HeLa cells (Fig. 5B). The evaluation of apoptosis measured by AnnexinV showed an increase in apoptotic cells in all HeLa cells when exposed to AS1411 (Fig. 5C). These data confirm that the over-expression of nucleolin in HeLa cells significantly increases cell sensitivity to AS1411 treatment. This most likely indicates that the increased nucleolin provides enhanced AS1411 uptake.

We have previously shown that AS1411 induces a cell cycle arrest at the S-phase.[1] Thus, it was important to evaluate the cell cycle characteristics of nucleolin overexpressing HeLa cells treated with AS1411. HeLa, HeLa-S7 and HeLa-SP cells underwent cell cycle analysis by flow cytometry before and after exposure to AS1411 for 72h. The data in Figure 6 show that treatment with 10 μ M of AS1411 perturbed the cell cycle progression in the non-transfected HeLa cells as well as the overexpressing clones. AS1411 leads to accumulation of cells in S-phase for HeLa and HeLa-S7 cells, while for HeLa-SP cells it appears that AS1411 induces predominantly a G1-phase arrest (Fig. 6A and 6B). This may reflect the different role played by nucleolin in the DNA replication and DNA repair mechanisms. Nevertheless, the accumulation of cells at either G1 or S phase of the cell cycle will reduce the relative number of dividing cells and eventually result in growth inhibition as observed in the MTT data. The cell cycle arrest and increase in apoptosis most likely result from the inhibition of nucleolin functions.

Nucleolin overexpression increases AS1411 uptake

In order to determine whether the increased AS1411 sensitivity of nucleolin overexpressing cells might reflect differential uptake of AS1411 oligonucleotide, we characterized the cellular internalization of fluorescently-labeled AS1411 (AS1411-Cy5) at three days. The resultant fluorescent micrographs demonstrated a significant increase in AS1411 oligonucleotide uptake by the HeLa-S7 and HeLa-SP that correlates with the increased expression of nucleolin (Fig 7A). In the nucleolin overexpressing cells, AS1411 is accumulated around and in the nuclei (arrows in Fig. 7A), while in HeLa and HeLa-pCMV AS1411 is more generally localized in the cytoplasm and around the nuclei. In addition, the increase in nucleolin expression in overexpressing (transfected) cells was confirmed by the high intensity of fluorescence in the HeLa-S7 cells. AS1411 uptake was quantitated by flow cytometry which demonstrated a marked increase uptake in nucleolin overexpressing cells HeLa-S7 (Fig 7B). This correlates with the increase in fluorescence intensity that was observed in the micrographs shown in Figure 7A. These data confirm that AS1411 binds to nucleolin, is shuttled through the cytoplasm to the nucleus. Furthermore, the increased nucleolin expression increases AS1411 uptake and consequently sensitizes the cells to AS1411.

Exposure of HeLa cells to AS1411 increases the expression of nucleolin

While analyzing cellular uptake of AS1411 by flow cytometry we also confirmed the increased expression of nucleolin. In this set of experiments, HeLa cells were exposed to

5 μ M of AS1411-AF488 labeled for 3 days then labeled with anti-nucleolin antibody to evaluate the presence of nucleolin. As expected, this experiment confirmed that HeLa-S7 and HeLa-SP cells expressed more nucleolin than HeLa and HeLa-pCMV (Fig 8A). Interestingly, however, the level of nucleolin expression in the non-transfected cells increased when they were exposed to AS1411, to achieve the level detected in HeLa-S7 or HeLa-SP (Fig 8A bottom panel and 8B). This observation was further confirmed by analyzing the total mRNA for nucleolin gene expression using RT-qPCR. The data in Figure 8C represent the fold change in nucleolin gene expression in treated cells compared to untreated. Nucleolin gene expression was increased >4 fold for HeLa and >5 fold for HeLa-pCMV when exposed to AS1411. While in nucleolin overexpressing cells HeLa-S7 and HeLa-SP there was less or no effect of AS1411 on nucleolin gene expression (Fig 8C). This is an interesting observation which suggests that nucleolin-binding compounds such as AS1411 have a significant effect on nucleolin gene expression. Altogether these observations suggest an important feedback loop which could explain the high efficiency of AS1411 in killing cancer cells.

Discussion

In this study, we demonstrated that cells overexpressing nucleolin become more sensitive to the cell death inducing effect of the G-quadruplex-forming oligonucleotide, AS1411. Interestingly, we have also shown, for the first time, that AS1411 induces increased expression of nucleolin, suggesting a potential feedback loop. Moreover, we confirmed the role of nucleolin in cancer maintenance as a more aggressive phenotype was observed in nucleolin overexpressing cells.

Nucleolin is overexpressed in actively dividing cells including cancer cells and therefore has been widely employed as a marker of cell proliferation, and in the designation of aggressive cancer as a component of the nucleolar AgNOR.[26, 31, 32] Although nucleolin is the most abundant protein of the nucleolus,[9] it is also found on the plasma membrane of tumor cells where it may act as a surface receptor for various ligands [20, 21]. Nucleolin has been shown to be a G-quadruplex binding protein, binding to G-quadruplex structures in the c-MYC promoter [5], in the HIV-1 LTR promoter,[47] and to AS1411[48]. We demonstrate herein that the amount of AS1411 internalized by HeLa cells correlates with the level of nucleolin expression.

AS1411 is the first anticancer aptamer to be tested in humans. It has been shown to have significant anticancer activity with very little toxicity in Phase I and Phase II clinical trials. [2] AS1411 was discovered by the serendipitous observation of its growth inhibitory activity against a wide variety of cancer cell lines.[2] Early work showed that its anticancer activity was related to its ability to form G-quadruplex for which nucleolin has high affinity. Although AS1411's exact mechanism of action is not known, it has been shown to bind to nucleolin present at the surface of cancer cells and to be actively taken up by macropinocytosis and subsequently transported to the nucleus of malignant cells.[49] The increasing uptake of AS1411 in cells overexpressing nucleolin such as cancer cells may explain the high intracellular concentrations of this molecule and its enhanced tumor cell toxicity.

AS1411's target protein, nucleolin, is a multifunctional phosphoprotein involved in a variety of key cellular processes including ribosomal biosynthesis, RNA binding, DNA and RNA helicase activity and intracellular localization of the telomerase complex.[8] Because of its multiple critical roles in regulating cell growth and proliferation, nucleolin is an excellent target for anti-proliferative therapies. Inhibition of nucleolin expression has been shown to cause decreased proliferation of cervical cancer cell lines[38] and cultured human primary fibroblasts[50]. AS1411 has been shown to induce cell cycle arrest,[50, 51] inhibit NF- κ B signaling,[52] inhibit BCL-2 signaling,[53] and to induce tumor suppressor gene expression.[54] Therefore, it is likely that AS1411 interferes with one or more of nucleolin myriad of biological functions.

The work presented here demonstrates that cells expressing higher levels of nucleolin have a greater sensitivity to growth inhibition by AS1411. When taken with the phenotypic changes induced by nucleolin overexpression which include increased proliferation and invasion, the increased sensitivity to growth inhibition induced by AS1411 suggests that it must be interfering with the intracellular activity of nucleolin, as suggested by Reyes-Reyes, et al. [49] While AS1411 mechanism of action has not been clearly defined yet, the increased cell death and the cell cycle arrest at G1/S phase observed in this study highly suggest that its binding to nucleolin prevent the induction of expression of proliferative and anti-apoptotic genes.[15, 55] Further study will be necessary to better define the exact mechanism involved in AS1411 activity. Interestingly, the presence of AS1411 also significantly induced nucleolin expression. This was especially apparent in cells in which nucleolin was not constitutively expressed and suggests the amplification of AS1411 effect in a feedback loop induction. This observation could explain the exponential increase in growth inhibition response of HeLa to AS1411 at 10 μ M compared to the response obtained with AS1411 at 5 μ M. The relationship between the level of nucleolin expression and the response to AS1411 has important implications for the future use of AS1411 or other DNA aptamers. It suggests that nucleolin overexpression may provide an effective biomarker for tumors that will be more sensitive to AS1411 treatment. That would also likely apply to oligonucleotides encoding genomic G-quadruplex-forming sequences which are in preclinical development. [56]

Several groups have shown that AS1411 is an excellent targeting vehicle to deliver either drugs[57–60] or nanoparticles[7, 61, 62] to tumor cells. Notably, AS1411 conjugated to nanoparticles has a markedly enhanced growth inhibitory effect when compared to AS1411 alone most likely because of increased stability of the molecule in the cytoplasm. Considering the data shown in this study, it is conceivable that the AS1411 moiety of the AS1411-drug-conjugated compounds will enhance the drug up-take/delivery exponentially and that doing so might circumvent the need for increased nucleolin expression to manifest maximal sensitivity.

Chemotherapy has been the primary systemic therapeutic approach for cancer treatment. Notably, Cisplatin constitutes the treatment of choice for cervical cancers (from which the HeLa cell line was derived). However, the incidence of drug-resistance is important and limits the application of this drug for long-term cancer treatment. HeLa cells are relatively resistant to Cisplatin treatment. We demonstrate here that HeLa cells overexpressing

nucleolin are more aggressive but only slightly more sensitive to treatment with Cisplatin or Camptothecin (only at low doses). Nucleolin overexpression can explain the mechanism by which certain tumor cells escape and develop a resistance to these treatments. Notably, nucleolin binds to topoisomerase I (target of Camptothecin) [63] and to the G-rich region of the DNA (especially G-quadruplex forming structures in the genomic DNA target of Cisplatin) which could explain the reduced effect of these drugs due to competition for their respective binding sites in the presence of nucleolin overexpression. Furthermore, nucleolin is involved in cell processes that favor proliferation and inhibit apoptosis [15] allowing the cells to escape the drug-induced apoptosis. Our finding that nucleolin overexpressing cells are more sensitive to AS1411 suggests a potential advantage in combined therapies using AS1411 either as carrier or as co-treatment with more conventional chemotherapeutic agents. Furthermore, the fact that AS1411 targets nucleolin will limit the toxicity of these drugs by aiming preferentially at cancer cells.

The growth inhibitory activity of AS1411 across a wide variety of tumor cell lines and the obvious role of nucleolin in its uptake and activity suggest that G-quadruplex-binding proteins, in general, may represent therapeutic targets. Genomic G-quadruplex-forming sequences occur in a variety of regions, including telomeric DNA[64] and immunoglobulin rearrangement sites[65]. These G-rich sequences are disproportionately represented in the promoters of human genes, particularly oncogenes; including the c-MYC[66], BCL-2[67] and VEGF genes.[68, 69] These sequences have been shown to be transcriptional silencers of several genes such as c-MYC or K-RAS.[69, 70] It is known that the G-quadruplex-forming sequence in c-MYC promoter and at the telomere both bind nucleolin and other G-quadruplex-binding proteins. It is therefore possible that one of the mechanisms of action of the exogenous G-quadruplex-forming oligonucleotides is to bind nucleolin and other G-quadruplex-binding proteins, interfering with their physiologic functions. In addition, the presence of nucleolin at the surface of cancer cells tags these cells for targeted therapy utilizing such molecules.

In conclusion, we have demonstrated that over-expression of nucleolin in HeLa cells confers increased sensitivity to growth inhibition induced by AS1411. This provides direct experimental corroboration for the critical role of nucleolin expression in AS1411 function. Although the exact mechanism by which AS1411 inhibits cellular proliferation remains unclear, it must involve interaction with nucleolin. Our data strongly support the exploitation of nucleolin expression as an important biomarker for AS1411 usage in future clinical trials. This would certainly be true of other G-quadruplex-forming aptamers as well.

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Figure 1: Analysis of HeLa cells overexpressing nucleolin.

A). Western blot analysis for nucleolin expression in HeLa overexpressing nucleolin clone #7 (S7) and pooled sense clones (SP) compared to empty vector transfected cells (pCMV) and non-transfected HeLa: representative gel and bar graph for quantification of the intact nucleolin bands (105–110KDa). B). Nucleolin gene expression measured by RT-qPCR expressed as fold change compared to untransformed HeLa cells. C). Proliferation of the nucleolin overexpressing cells compared to HeLa at 24 and 48h measured by MTT assay. Data represent the average for three individual experiments, * for p values < 0.05 .

Figure 3: Nucleolin overexpression slightly increases HeLa cells sensitivity to chemotherapeutic agents.

Cell proliferation measured by MTT assay on HeLa, HeLa-pCMV, HeLa-S7 and HeLa-SP at 3-day culture A). Cisplatin (1 to 50 μ M) and B). Camptothecin (10nM to 10 μ M). Data represent the average of three individual experiments, * for p values < 0.05 when compare each cell line treated to untreated, # for p values < 0.05 when compared transfected to non-transfected HeLa cells.

Figure 4: Overexpression of nucleolin sensitizes HeLa cells to AS1411.

HeLa, HeLa-S7 and HeLa-SP cells were set in culture in the presence of AS1411 at different doses (from 1μM to 10μM) for 3 days and evaluated for proliferation in MTT assay. Data represent the average absorbance normalized to the control untreated for three separate experiments, * for p values < 0.05 when compare each cell line treated to untreated, # for p values < 0.05 when compared transfected to non-transfected HeLa cells, § for $p < 0.05$ when comparing HeLa treated with AS1411 at 5μM and 10μM.

Figure 5: AS1411 induces cell death and apoptosis in HeLa and overexpressing Nucleolin HeLa cells

HeLa, HeLa-S7 and HeLa-SP cells were set in culture in the presence of AS1411 at 10 μ M for 3 days and evaluated for: A). cell growth and B). cell death by Trypan blue exclusion assay. C). Apoptosis assay using AnnexinV/7AAD and measured by FACS analysis. Data represent the average cell count for three separate experiments, * for p values <0.05 when compared transfected to non-transfected HeLa cells.

Figure 6: AS1411 induces cell cycle arrest at G1 and S phases in HeLa and HeLa overexpressing Nucleolin.

HeLa, HeLa-S7 and HeLa-SP cells were set in culture in the presence of AS1411 at 10 μ M for 3 days then fixed. The DNA content was evaluated by 7AAD and measured by Flow-cytometry. A). Representative flow-cytometry histograms for HeLa, HeLa-S7 and HeLa-SP untreated (top) and AS1411 treated cells (bottom) showing the accumulation of the cells in S-phase or G1-phase. B). Bar graph for the average of the percent of cells present in each phase of the cell cycle. Data represent the average $\pm$ SE for three separate experiments.

Figure 7: Overexpression of nucleolin in HeLa cells increases the cellular up-take of AS1411
 A). Cellular uptake for AS1411-Cy5-labeled (5 μ M) in HeLa, HeLa-pCMV, HeLa-S7 and HeLa-SP after 72h exposure (red) nuclear localization is indicated by the white arrows (and in inset). Expression of nucleolin was revealed with an antibody anti-nucleolin/and secondary -AF488 (green), DAPI (blue) was used for nuclear staining. Pictures were taken at 200x magnification. B). figure shows flow-cytometry histograms for the evaluation of AS141-FAM labeled (5 μ M) up-take in HeLa, HeLa-pCMV, HeLa-S7 and HeLa-SP in one representative experiment.

Figure 8: AS1411 treatment increases nucleolin expression in HeLa cells

A). Measure of nucleolin in HeLa, HeLa-pCMV, HeLa-S7 and HeLa-SP untreated and exposed to 5 μ M of AS1411 by flow-cytometry. Figure show one representative experiment, B). Bar graph represents the average of nucleolin positive cells for two separate experiments. C). Bar graph to represent the effect of AS1411 treatment on nucleolin gene expression measured by RT-qPCR. Data represent the average of three experiments.