SUPPORTING INFORMATION

Grafting aptamers onto gold nanostars increases in vitro efficacy in a wide range of cancer cell types

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MATERIALS AND METHODS

Cell culture: All cultures were grown in a humidified incubator maintained at 37 °C with 95% air/5% CO2. All cell lines were obtained from the American Type Culture Collection. HT-10180 (connective tissue), U-87 (brain), DU-145 (prostate), SK-MEL-2 (skin), and A-498 (kidney) cancer cells were cultured in MEM (Invitrogen) growth medium supplemented with 10% fetal bovine serum (FBS) (Invitrogen). HCT-116 (colon), MDA-MB-231 (breast) and SKOV-3 (ovarian) cancer cells were grown in RPMI 1640 (Invitrogen) medium supplemented with 10% FBS. A-549 (lung) and MCF-7 (breast) cancer cells were maintained in DMEM/F12 (Invitrogen) medium supplemented with 10% FBS. PANC-1 (pancreas) and HeLa (cervix) cancer cells were cultured in DMEM (Invitrogen) growth medium with 10% FBS. Fibroblast cells HS-27 (skin) and WI-38 (lung) were propagated in DMEM and MEM complete growth media, respectively, supplemented with 10% FBS. MCF-10A (normal human mammary epithelial) cells were cultured in DMEM/F12 growth medium supplemented with 5% horse serum (Invitrogen), 20 ng/mL epidermal growth factor (Sigma-Aldrich), 0.5 mg/mL hydrocortisone (Sigma-Aldrich), 100 ng/mL cholera toxin (Sigma-Aldrich), and 10 µg/mL insulin (Sigma-Aldrich).

Synthesis of AuNS: Gold nanostars (AuNS) were synthesized by reducing Au (III) chlorate in HEPES buffer to create biocompatible, surfactant-free gold nanoparticles for in vitro studies. The AuNS were prepared by mixing 5 µL of 40 mM HAuCl4 (Sigma Aldrich) with 1 mL of 140 mM 2-[4-(2-hydroxyethyl)piperazine-1-yl]ethanesulfonic (HEPES) buffer.\(^1\) The resonance wavelength of the AuNS was measured using UV-Vis spectroscopy. Particle size was determined using transmission electron microscopy (TEM) and dynamic light scattering (DLS).
**Preparation of Apt-AuNS nanoconstructs:** The synthesis of Apt-AuNS was performed as previously reported.\(^1\) AS1411 aptamer with a disulfide modification at the 5’-end (5’-T-(C6-S-S-C6)-TTG GTG GTG GTT GTG GTG GTG GTG G-3’) was purchased from IDT DNA, Inc. HPLC-purified aptamers were dissolved in Millipore water (18.2 MΩ cm) to obtain 1 mM solutions. The disulfide bond was cleaved by adding 2.5 µL of 25 mM tris(2-carboxyethyl)phosphine (TCEP) (Sigma Aldrich) to 10 µL of the 1 mM aptamer solution. After 30 min, the thiolated aptamer solution was added to 10 mL of 0.19 nM AuNS and left overnight to form the nanoconstruct (Apt-AuNS). To increase the surface concentration of aptamers on AuNS, we salted the mixture solution with 2.5 mL of a 500 mM solution of NaCl twice, separated by 4 h.

**Quantifying number of AS1411 strands on AuNS:** Cy5-labeled aptamer 5’-(C6-S-S-C6)-Cy5-TTG GTG GTG GTT GTG GTG GTG GTG G-3’ (Cy5-Apt) was used to estimate the number of aptamers on each particle. Attachment of Cy5-Apt to the AuNS followed the same procedure as described previously. We centrifuged 200 µL of Cy5-labeled Apt-AuNS (Cy5-Apt-AuNS) at 15,000 rpm for 11 min. The supernatant was removed, and the nanoconstructs were suspended in 1 mL of 50 mM HEPES buffer. This process was repeated twice to eliminate unbound Cy5-Apt. Cy5-Apt-AuNS pellets were treated with 100 µL of 20 mM potassium cyanide (KCN) overnight to dissolve the Au core of the nanoconstruct and release Cy5 aptamer into the solution. The Cy5 fluorescence intensity of the KCN solution was measured using a NanoDrop Spectrophotometer, and the concentration of AS1411 was determined based on the intensity of the Cy5 signal. This fluorescent assay indicated that approximately 110 strands of AS1411 were conjugated on a single AuNS.
**Immunoblotting of nucleolin:** Cancer and normal cells were harvested from cell culture flasks. To determine expression of cytosolic nucleolin in cancer and normal cells, we lysed the cells on ice for 15 min in 5% NP40 lysis buffer (Invitrogen), followed by centrifugation at 10,000 g for 20 min. The supernatant contained the cytosolic extract, and the pellet contained the nuclear components.

For plasma membrane extraction, cancer and normal cells were lysed in Homogenize Buffer (Abcam) and homogenized 50 times on ice using a Dounce homogenizer. The nuclei were removed by centrifugation at 700 g for 10 min at 4 °C. The supernatant was centrifuged at 10,000 g for 30 min at 4 °C. After centrifugation, the new supernatant containing the cytosol fraction was removed. The pellet containing the total cellular membranes was purified to isolate further the plasma membranes from other organelle membranes. The total cellular membrane pellet was re-suspended in mixture of Lower Phase and Upper Phase solutions (Abcam). The solution was centrifuged at 1,000 g for 5 min at 4 °C. The upper phase was collected and diluted with nuclease free water (Invitrogen). The diluted solution was centrifuged at 21,000 g for 20 min at 4 °C. The resultant pellet contained the plasma membrane proteins.

Protein concentrations after the extractions were determined by the Bradford assay (Pierce). Aliquots of the cytosolic extracts containing 20 µg of protein were electrophoresed on a 10% Tris-HCl gel (Bio Rad) and transblotted using PVDF membranes (Millipore). Antihuman nucleolin mAb (clone MS-3) (Santa Cruz Biotechnology, Inc) and anti-mouse actin polyclonal antibody (Sigma-Aldrich) were used to label nucleolin and the housekeeping protein β-actin. The blot was stained with Alkaline Phosphatase-IgG secondary antibody. The bands were developed using enhanced chemifluorescence substrate (GE Healthcare) and visualized by Typhoon PhosphorImager (GE Healthcare). The amount of each protein in the blots was determined by
counting the total number of pixels in each band (integrated density value) with ImageJ. Values of nucleolin that were within the linear range of the assay were normalized to β-actin for the cytosolic extracts.

Confocal imaging of Cy5-labeled nanoconstructs: Each cell line was plated on collagen I coated coverslips (BD Biosciences) (20,000 cells/cover slip) and cultured in their respective complete growth media. After 24 h of cell growth (37 °C, 5% CO2), the media was replaced with fresh growth media containing 0.3 nM Cy5-labeled Apt-AuNS. Cells were incubated with the nanoconstructs for 7 h and then washed three times with PBS. The cells were fixed with 4% paraformaldehyde (Sigma Aldrich) for 15 min followed by three washes with PBS. A drop of ProLong Gold Antifade reagent containing DAPI (Invitrogen) was used to mount each coverslip on a glass slide for confocal imaging. Confocal imaging was performed on an inverted Zeiss Axio Observer Z1 confocal microscope with a 40x objective and Zen acquisition software.

Quantification of nanoconstruct uptake in cells: Cells were plated in 12-well plates (~60,000 cells/well) for 24 h and then 0.3 nM Apt-AuNS in growth media was added to each well. The cells were incubated with the nanoconstructs for 7 h at 37 °C in 5% CO2 environment. After 7 h, excess Apt-AuNS were removed from the wells, and the cells were washed twice with ice cold PBS (Invitrogen). The cells were then harvested and suspended in 100 µL PBS. The cells were counted using a hemocytometer before being digested for 4 h at 75 °C in acid mixture containing 200 µL of 30% HCl (Sigma-Aldrich) and 86 µL of 70% HNO3 (Sigma-Aldrich). After complete digestion of the AuNS, the solution was diluted with Millipore water to a final volume of 6 mL. The Au content was measured using ICP-MS.

Ultra-fast light triggered release of aptamers from AuNS. Femtosecond, near-infrared (NIR) pulses generated using a 1 kHz Ti:sapphire regenerative amplifier (Spitfire Pro, Spectra-Physics)
seeded by a Ti:sapphire oscillator (Tsunami, Spectra-Physics) were used to release Apt from AuNS. The center wavelength was set to 800 nm, and the pulse duration was 40-50 fs. The beam was focused to a 4.5-mm diameter spot at the sample using a 50 cm focal length plano-convex lens. The average pump power was attenuated to 760 mW, corresponding to a power density of 4.8 W/cm², using a gradient neutral density filter wheel. Samples were irradiated in the 96-well plates, and the irradiation time was controlled to within 2 ms by a shutter (Uniblitz, Vincent Associates) connected to a computer.

**Quantification of BCL-2 mRNA expression:** Real-time polymerase chain reaction (qPCR) was used to quantify mRNA expression in cancer and normal cells. The assay was conducted using Power SYBR Green Cells-to-Ct Kit (Invitrogen). 106 cells were harvested from cell culture flasks and were washed with 0.5 mL of cold PBS three times. The cells were centrifuged at 1,000 g for 5 min. The cell pellets were resuspended in fresh cold PBS to a concentration of 2,000 cells/µL. 5 µL of the cell solution was distributed to wells of a 96-well U-bottom plate (VWR) and situated on ice. 50 µL of lysis solution containing DNase I (Sigma-Aldrich) was added and mixed with the cell solution in each well. The lysis reaction was incubated for 5 min at room temperature. 5 µL of stop solution (Invitrogen) was added to each well and incubated for 2 min to stop the lysis reaction. Each RNA sample was then quantified with a NanoDrop Spectrophotometer, and the RNA integrity of each sample was confirmed by assessing the ratio of two major mammalian ribosomal RNAs 28S and 18S. All samples exhibited 28S/18S RNA ratios of 1.8–2.2, which indicates the presence of high-quality total RNA.

RNA lysates were reverse transcribed to synthesize cDNA. The reverse transcription (RT) process was done by combining 10 µL of RNA lysates, 25 µL of 2X SYBR RT buffer (Invitrogen), 2.5 µL of 20X RT Enzyme mix (Invitrogen) and 12.5 µL of nuclease-free water.
The mixture was run through the RT thermal cycler program, incubated at 37 °C for 60 min and then at 95 °C for 5 min to inactivate the RT enzyme. The resultant cDNA solution was stored at -20 °C for qPCR.

Primers for qPCR were designed using freely available software from IDT DNA. Identified primers were purchased from IDT DNA and tested for the amplification of a single uniform amplicon through analysis of SYBR melting curves for two cell lines (HeLa and A-549). In addition, negative controls for each primer pair were run in the absence of cDNA template to ensure that all readings were the result of the intended amplification reaction. For Bcl-2 mRNA, the reverse primer was 5’–AGG TCT GGC TTC ATA CCA C AG GTT– 3’ and forward primer 5’–TTT CTC ATG GCT GTC CTT CAG GGT–3’. For ACTB (β-actin) mRNA, the reverse primer was 5’–AGG ATG GCA AGG GAC TTC CTG TAA–3’ and forward primer 5’–AAT GTG GCC GAG GAC TTT GAT TGC– 3’.

All qPCR reactions were performed in 20-µL reaction mixtures containing 4 µL of cDNA, 10 µL Power SYBR green PCR Master Mix (Invitrogen), 0.15 µL each of 400 nM forward and reverse primers, and 5.7 µL nuclease-free water. Each sample was run in triplicate on a 96-well U-bottom plate with the IQ5 qPCR system (Bio-Rad). Each plate probed the expression of Bcl-2 and ACTB genes in the target cell lines. Thermo-cycling conditions consisted of an enzyme activation step at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, 60 °C for 1 min. Afterwards, melting curves were generated to confirm a single uniform peak. Baselines were automatically set by the software, and the threshold was manually set to a single value of 500 for all experiments. Real-time PCR data were analyzed using the comparative CT method, also known as the \( 2^{-\Delta\Delta C_T} \) method, in which the expression of Bcl-2 mRNA was normalized to the expression of the housekeeping gene ACTB. The S.E.M. values for the 9 replicates for each gene
in all 15-cell lines (12 cancer cell lines and 3 normal cell lines) were calculated from these normalized C_T values.

**Measurement of caspase 3/7 activities:** Apo-ONE Homogeneous Caspase-3/7 assay kit (Promega) was used to test caspase 3/7 activity, an apoptotic indicator, in cancer and normal cells after treatment with Apt-AuNS, Apt-AuNS + hν and 450 nM free AS1411. The cells were plated on a half-area 96-well plate 24 h in advance. The number of cells plated depending on the doubling time of the cell line so that the population was not over-confluent at 72-h. After treatment with Apt-AuNS, the cells were lysed with a mixture containing pro-fluorescence substrate (Z-DEVD-R110) and bifunctional cell lysis/activity buffers for 1 h at room temperature. We measured the caspase 3/7 activity using a Synergy 3 microplate reader to determine the fluorescence intensity of R110 (487_ex/528_em nm) at different time points (0 h and 72 h) after treatment. Error bars were calculated by taking the ratio between standard deviation of the sample set and square root of the sample population (N = 12). One-way ANOVA test was performed (Origin, 8th ed.) to evaluate significant differences among the different treatments in the same cell line. One-way ANOVA measures the significant effect of one independent variable, while two-way ANOVA is used when there is more than one independent variable and multiple observations of each independent variable.

**Measurement of cell viability:** Cell-Titer Blue Cell viability assay (Promega) was used to determine the viability of cells. Viable cells reduce non-fluorescent rezasurin to resofurin, which is a highly fluorescent molecule while nonviable cells do not reduce rezasurin. Cells were plated on a half-area 96-well plate 24-h in advance. The number of cells plated depending on the doubling time of the cell line so that the population was not over-confluent at 72-h. After treatment, cells were subjected to a cell-titer blue solution for 3 h at 37 °C and 5% CO₂. We
determined cell viability by measuring the fluorescence intensity (560_{ex}/ 590_{em} nm) of resofurin at different time points (0 h and 72 h). Error bars were calculated by taking the ratio between standard deviation of the sample set and square root of the sample population (N = 12). One-way ANOVA test was performed (Origin, 8\textsuperscript{th} ed.) to evaluate significant differences among the different treatments in the same cell line.
Figure S1: Localized surface plasmon (LSP) resonance of gold nanostars (AuNS). The LSP resonance of AuNS (black line) was within the biological transparent window and centered around 800 nm, while the Apt-AuNS (red line) has LSP resonance slightly red-shifted. Femtosecond pulsed laser excitation at the LSP wavelength could excite the AuNS to trigger the release of aptamers (Apt) from the surface of the gold nanoparticles. The LSP resonance of Apt-AuNS after two months in water (blue line) indicated no significant changes of the nanoconstruct.
Figure S2: Nucleolin expression in the plasma membranes of cancer and normal cells. Nucleolin levels in the plasma membrane of HeLa (cervical cancer) and HT-1080 (fibrosarcoma) were much higher than that in HS-27 (fibroblast) cells.
Figure S3: Elevation of caspase activities in cancer cells after treatments. The caspase 3/7 activities increased up to 3 times in cancer cell immediately (t = 0 h) after treatment with Apt-AuNS + \( h_\nu \). These protease activities only elevated \( ca. \) 1.3 times after treatment with Apt-AuNS. The elevation of caspase activities was significant lower in cancer cells treated with free 450 nM AS1411 compared to that in Apt-AuNS + \( h_\nu \). No significant increase in caspase activity was observed in normal cells. P-values were determined using one-way ANOVA test. Lines over bars indicate groups that are not significantly different.
Figure S4: Cell viability of cancer and normal cells after treatments. There was \( ca. 30\% \) increase in the amount of cancer cell death at \( t = 0 \) h after treatment with \( \text{Apt-AuNS + } h\nu \) (\( \sim 52\% \)) compared to \( \text{Apt-AuNS} \) (\( \sim 82\% \)). Only 8\% cell death on average was observed in cancer cells immediately after treatment with 450 nM free AS1411. No significant cell death was observed in normal cells. p-values were determined using one-way ANOVA test. Lines over bars indicate groups that are not significantly different.
Figure S5: Calibration curve of Cy5-Apt. A linear calibration curve of Cy5-AS1411 concentration and Cy5 fluorescence intensity was constructed to determine the amount of Cy5-AS1411 on the surface of the AuNS.
Table S1: Summary of biological assay results in the 12-cancer cell line panel at $t = 72h$

|                         | Apt-AuNS + h | Apt-AuNS | 450 nM AS1411 |
|-------------------------|--------------|----------|---------------|
| **Average BCL-2 mRNA**  |              |          |               |
| **reduction**           | 362%         | 224%     | 150%          |
| **(min-max)**           | (130%) - (150%) | (110%) - (430%) | (100%) - (400%) |
| **(DU-145) (PANC-1)**   | (DU-145) (PANC-1) | (DU-145) (PANC-1) | (SKOV-3) (PANC-1) |
| **Average elevation of**|              |          |               |
| **caspase 3/7 activity**| 3.35         | 1.58     | 1.16          |
| **(min-max)**           | (1.7) - (5.5) | (1.0) - (3.8) | (0.9) - (1.5) |
| **(HeLa) (HCT-116)**    | (HeLa) (HCT-116) | (SKOV-3) (HCT-116) | (U-87) (HeLa) |
| **Average percentage of**|              |          |               |
| **cell death**          | 65%          | 27%      | 10%           |
| **(min-max)**           | (55%) - (78%) | (0%) - (35%) | (0%) - (15%) |
| **(SKOV-3) (A-498)**    | (SKOV-3) (U-87) | (SKOV-3) (U-87) | (SKOV-3) (A-549) |
Table S2: Information of cancer cell lines

| Cell Line   | Organ             | Type   | Doubling Time (h) |
|------------|-------------------|--------|-------------------|
| HCT-116    | Colon             | Carcinoma | 17.4 $^3$        |
| HT-1080    | Connective Tissue | Fibrosarcoma | 18.2 $^4$       |
| A-549      | Lung              | Carcinoma | 22.9 $^3$        |
| HeLa       | Cervix            | Carcinoma | 24 $^5$          |
| MCF-7      | Breast            | Carcinoma | 25.4 $^3$        |
| U-87       | Brain             | Glibastoma | 29.8 $^6$        |
| DU-145     | Prostate          | Carcinoma | 32.3 $^3$        |
| MDA-MB-231 | Breast            | Carcinoma | 41.9 $^3$        |
| SK-MEL-2   | Skin              | Melanoma | 45.5 $^3$        |
| SKOV-3     | Ovary             | Carcinoma | 48.7 $^3$        |
| PANC-1     | Pancreas          | Carcinoma | 52 $^7$          |
| A-498      | Kidney            | Carcinoma | 66.8 $^3$        |
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