Electronic Supplementary Material (ESI) for Dalton Transactions.

Supporting Information for

Aptamer-Mediated Selective Delivery of a Cytotoxic Cationic NHC-Au(I) Complex to Cancer Cells

Weijia Niu, I-Ting Teng, Xigao Chen, Weihong Tan, and Adam S. Veige*

University of Florida, Department of Chemistry, P.O. Box 117200,

Gainesville, FL, 32611

Contents

1. General consideration

Unless stated otherwise all synthesis and manipulations were performed under aerobic conditions. $(CH_3)_2SAuCl$, Ag_2O, 1-methylbenzimidazole, 9-(chloromthyl)anthracene, pentrafluorophenol, 4-(bromomethyl)benzoic aicd , potassium hexafluorophosphate and triphenylphosphine were obtained from Sigma Aldrich and used without further purification. ^{1}H, ^{19}F, ^{31}P and ^{13}C NMR spectra were obtained on Varian INOVA spectrometer (500 MHz), or a Mercury (300 MHz). Chemical shifts, reported in δ (ppm), were referenced on the solvent, on the TMS scale for ^{1}H and ^{13}C. The emission spectra of compounds were measured using a HORIBA Jobin Yvon fluorescence spectrophotometer. Elemental analyses were performed at Robertson Microlit Laboratories., Ledgewood, New Jersey. The purification step was performed by HPLC (ProStar, Varian, Walnut Creek, CA, USA) with a C18 column (5 μm, 250 mm × 4.6 mm, Higgins Analytical) using acetonitrile and 0.1 M triethylammonium acetate (TEAA) aqueous solution as the mobile phase. UV−Visible absorption measurements were carried out on Varian Cary 100 UV-Vis spectrometer (Agilent Technologies, Santa Clara, CA, USA).

2. DNA synthesis

All oligonucleotides were synthesized on an automated ABI3400 DNA/RNA synthesizer (Applied Biosystems, Foster City, CA, USA) through solid phase oligonucleotide synthesis starting from corresponding controlled pore glass (CPG) at a 1 μmol scale.[1] Amine was directly coupled at the 5′-end of oligonucleotides with an extended coupling time. All oligonucleotides were deprotected in 3 mL AMA solution (ammonium hydroxide: 40% aqueous methylamine = 1 : 1) at 65 °C for 30 min. Then the oligonucleotides were precipitated by adding 250 μL of 3 M NaCl and 6 mL of cold ethanol. Then the precipitated oligonucleotides were collected by centrifugation at 4000 rpm for 30 min and dissolved in 400 μL of triethylammonium acetate (TEAA) for further purification by reverse-phase high-pressure liquid chromatography (HPLC) (ProStar, Varian, Walnut Creek, CA, USA) with a C18 column (5 μm, 250 mm × 4.6 mm, Higgins Analytical) using acetonitrile and 0.1 M triethylammonium acetate (TEAA) aqueous solution as the mobile phase. After being dried by SpeedVac concentrator, the oligonucleotides products were obtained, and their concentrations were measured with a UV−vis spectrometer (Cary Bio-300, Varian).

Type	Sequence
AS1411	5’- TTT TTT GGT GGT GGT GGT TGT GGT GGT GGT GG TTT TTT FITC-3’
AS1411-NH_2	5’-NH_2 TTT TTT GGT GGT GGT GGT TGT GGT GGT GGT GG TTT TTT FITC-3’
LIB-NH_2	5’-NH_2 TTT TTT NNN NNN NNN NNN NNN NNN NNN NNN NN TTT TTT FITC-3’

Table S 1. DNA Sequences designed in this work.

3. Cell culture

All cell lines used in this research (other than Hu1545v cell) were purchased from American Tissue Culture Collection (ATCC). Hu1545v cell line was established by immortalizing primary hepatocyte (normal liver cell) with lentivirus carrying HTERT (human telomerase reverse transcriptase, the enzyme maintains telomere length at the end of chromosomes thus enables cells to grow and proliferate). The cell was equipped with extended lifespan and remains the characteristic protein profile of normal liver cells.[2] All cell lines were cultured in American Tissue Culture Collection recommended medium, with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and 0.5 mg/mL penicillin-streptomycin (American Type Culture Collection) at 37 °C under a 5% CO_2 atmosphere, except for MDA0MB-231, which was cultured without CO_2. CCRF-CEM (T-cell line) was cultured in RPMI 1640 medium; MDA-MB-231 (breast cancer cell) was cultured in L-15 medium; DU145 (prostate cancer cell) was cultured in EMEM medium; Hela (cervical cancer cell), HEK293 (human kidney cell) and HU1545v (human liver cell) were cultured in DMEM medium. Cells were washed before and after incubation with washing buffer [4.5 g/L glucose and 5 mM $MgCl_2$ in Dulbecco's PBS with calcium chloride and magnesium chloride (Sigma-Aldrich)]. Binding buffer was prepared by adding yeast tRNA (0.1 mg/mL; Sigma-Aldrich) and BSA (1 mg/mL; Fisher Scientific) to the washing buffer to reduce background binding. All reagents for buffer preparation and HPLC purification came from Fisher Scientific. Unless otherwise stated, all chemicals were used without further purification.

4. MTS assay

The cytotoxicity of NHC-Au complex or aptamer-drug conjugate for each individual type of cell was determined by MTS assay using a CellTiter 96 cell proliferation assay (Promega, Madison, WI). About 1×10^4 of cells were suspended in medium and were seeded into 96-wells plates and cultured for 24 h. Then the medium was replaced with fresh medium (no fetal bovine serum) containing the NHC-Au complex or the prepared aptamer-drug conjugate at a series of desired concentrations. Cells were further incubated for 4 hours to allow the uptake of the drug and aptamer drug conjugate. After which, cells were washed twice with PBS buffer and supplemented with fresh medium to allow another 48-hour incubation. For all the cells, after 48 h incubation, the supernatant was removed, and then CellTiter reagent (20 μL) diluted in fresh medium (100 μL) was added to each well and incubated for 1−2 h. The absorbance (490 nm) was recorded by using a plate reader (Tecan Safire microplate reader, AG, Switzerland). Each cell measurement had the treatment background subtracted before analysis. Cells in media alone was used as a control. The IC_{50} values were calculated as the concentrations reducing proliferation of untreated control cells by 50% and are given as the means and errors of 3-5 independent experiments. Quantitative and statistical analyses were performed using the nonlinear dose response curve fitting in Origin 8.5 software.

5. Flow cytometry analysis

Cells were plated in a 75 cm^2 tissue culture flask (Olympus Plastics, Genesee Scientific, CA, USA) and grown to around 80% confluency for 24 h before the experiments. Cells were washed twice with 1 mL PBS and dissociated using nonenzymatic dissociation buffer, then incubated with the prepared aptamer-drug conjugate at the concentration of 250 nM for 30 min at 4°C. After incubation, cells were washed with washing buffer three times, dispersed in 80 μL binding buffer, and finally subjected to flow cytometry analysis using BD Accuri C6 cytometer (Becton Dickinson Immunocytometry System, San Jose, CA). Fluorescence was determined by counting 30,000 events, and data were analyzed with FlowJo software. AS1411 aptamer was used as a positive control at the same condition, and LIB-**2** conjugate was used as negative control to determine the background binding.

6. Confocal microscopy

Cells were incubated with 100 nM AS1411-**2** conjugate in serum-free cell culture medium at 37°C with 5% CO_2 for 30 min. Then the cells were washed three times with Dulbecco's PBS and taken for confocal microscopy. In the internalization experiment, HeLa cells and HEK293 cells were incubated with 100 nM AS1411-**2** conjugate in serum-free cell culture medium at 37°C with 5% CO_2 for 2h, followed by stained with diluted Hochest 33342 (1:10000 dilution) solution for another 10 min. Then the cells were washed three times with Dulbecco's PBS. Fluorescence imaging was performed on a Leica TCS SP5 confocal microscope (Leica Microsystems) with a 63× oil immersion objective. AS411-**2** was excited at 460 nm. Hoechst 33342 was excited at 405 nm.

7. Gel electrophoresis

Each DNA sample (10 μM, 10 μL) was mixed with 4 μL of glycerol and analyzed by 3% agarose gel at 110V for 25 min in 1x TBE buffer (89 mM tris(hydroxymethyl)aminomethane, 2 mM ethylenediamine tetraacetic acid and 89 mM boric acid, pH 8.0). The bands were stained by ethidium bromide (EB), visualized by UV illumination (312 nm), and photographed by a digital camera.

8. Synthesis of NHC-Au complex 2

100 mg of complex **1** was dissolved in 5 mL of acetone, then 35.0 mg of triphenylphophine (0.133 mmol, 1.10 equiv.) and 24.0 mg of KPF_6 (0.133mmol, 1.10 equiv.) were added. The mixture was stirred at room temperature for 30 min. Then the mixture was filtered through Celite and the filtrate was dried under vacuum. The yellow residue was dissolved in 2 mL of DCM and washed several times with water, then 10 mL of hexane was added to the organic solution to precipitate the cationic NHC-Au complex **2**. Complex **2** was purified by washing with hexane for 3 times (0.112 g, yield=81.7%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.21 (d, 2H, $^{3}J_{HH}$ = 8.7 Hz, *H*Ar), 8.05 (s, 1H, *H*Ar), 7.94 (d, 2H, $^{3}J_{HH}$ = 8.3 Hz, *H*Ar), 7.79 (d, 2H, $^{3}J_{HH}$ = 8.7 Hz, *H*Ar), 7.55 (m, 8H, *H*Ar), 7.41 (m, 8H, *H*Ar), 7.16 (d, 2H, $^{3}J_{HH}$ = 8.3 Hz, *H*Ar), 6.84 (dd, 6H, $^{3}J_{HH}$ = 12.7, 7.6 Hz, *H*Ar), 6.56 (s, 2H, CH_2-antrancene), 5.86 (s, 2H, CH_2-benzyl) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 190.14 (*C*-Au), 162.00 (*C*=O), 142.56 (*C*-Ar), 138.49 (*C*-Ar), 138.03 (*C*-Ar), 134.11(*C*-Ar), 131.39 (*C*-Ar), 129.40 (*C*-Ar), 127.56 (*C*-Ar), 125.42 (*C*-Ar), 123.05 (*C*-Ar), 112.27 (*C*-Ar), 52.12 (*C*H_2-antrancene), 42.09 (*C*H_2-benzyl) ppm. ^{31}P NMR ($CDCl_3$, 121 MHz): δ = 106.10 (q, *P*F6), 38.34 (s, Au-*P*Ph3) ppm. ^{19}F NMR ($CDCl_3$, 282 MHz): δ = –152.51 (t, $^{3}J_{FF}$ = 19.1 Hz, 2F, C*F*), –157.59 (t, $^{3}J_{FF}$ = 23.2 Hz, 1F, C*F*),–162.15 (dd, $^{3}J_{FF}$ = 19.1, 23.2 Hz, 2F, C*F*) ppm. DART-MS (positive ion, for M^+ = $C_{54}H_{36}AuF_5N_2O_2P$). $[M]^+$ calcd 1067.2095; Found: 1067.2101. Elemental analysis calcd. (%) for $C_{54}H_{36}AuF_{11}N_2O_2P_2$ (1212.17g/mol): C: 53.48, H: 2.99, N:2.31; Found: C: 53.70, H: 3.28, N: 2.39.

9. Synthesis of aptamer-drug conjugate

In a 2 ml eppendorf ® tube, 0.1 μmol (1 equiv.) of aptamer on solid supporting beads were suspended in a 1000 μL THF solution of NHC-Au complex **2** (10 μmol, 100 equiv.). 1.4 μL of trimethylamine (100 equiv.) was slowly added and completely mixed. The mixture was shaken at room temperature for 18 h, after which it was centrifuged for 30 minutes at 4,000 rpm. The supernatant was decanted and the solids part (crude DNA products on the beads) were washed three times with THF, then collected and transferred to a new test tube. The crude DNA product were mixed with 2000 μL AMA (Ammonium Hydroxide, methylamine 50:50) and incubated at 65˚ for 30 minutes. Wait until the deprotection solution cools down, transfer the supernatant (leaving all the cpg beads behind) into a plastic centrifuge tube. 250 μL of 3M NaCl solution and 6000 μL of cold ethanol were added to the tube, then the mixture was vortexed and DNA slowly precipitated. After 1 hour freezing, the mixture was centrifuged for 30 minutes at 4,000 rpm and supernatant discarded. 400 μL 0.10 M TEAA (triehtylamine acetate) was added to the same tube to dissolve (with the help of pipette tip) the solids left from last step. After all the DNA dissolved, transfer the solution to an Eppendorf tube for HPLC purification. HPLC condition: Eluent A: 0.10M TEAA aqueous solution; Eluent B: acetonitrile. Low pressure gradient by varying B concentration from 10% (min 4) to 66 % (min 42:15), and then to 100 % (min 47:37). The flow rate was kept at 1 ml/min, and temperature was maintained at room temperature. AS1411-**Ligand** and LIB-**2** were synthesized with the same

method.

10. Quantification of **AS1411-2** by using UV-visible spectroscopy.

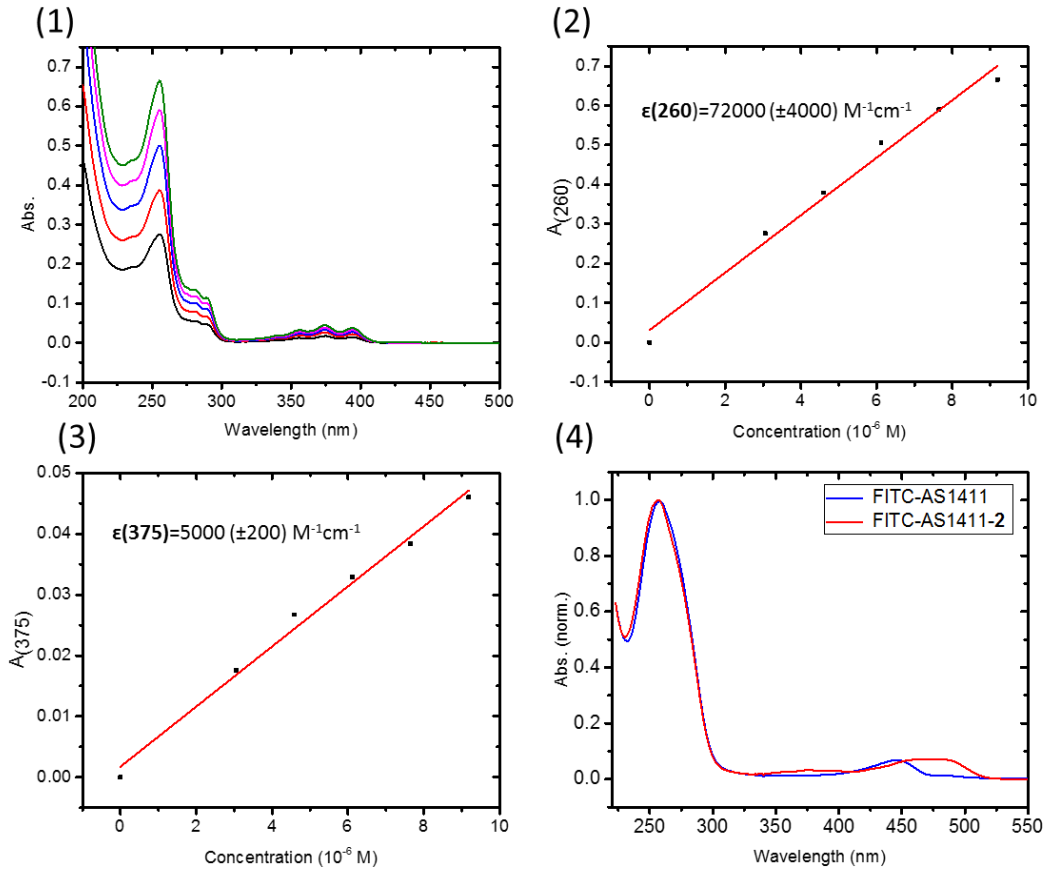

Figure S 1. (1) UV-visible absorption spectra of NHC-Au complex **2** at different concentrations (H_2O: Acetonitrile=1:1). (2) The molar extinction coefficient of NHC-Au complex **2** was determined using Lambert-Beer law with a result of ε (260 nm) =72000 $M^{-1}cm^{-1}$. (3) The molar extinction coefficient of NHC-Au complex **2** was determined using Lambert-Beer law with a result of ε (375 nm) =5000 $M^{-1}cm^{-1}$. (4) UV-visible absorption spectra of AS1411-NHC-Au conjugate and AS1411, the absorbance at 260 nm for both sample was normalized to 1 (H_2O: Acetonitrile=1:1).

The UV-visible absorption spectra of complex **2** was collected at five concentrations from 3.6×10^{-6} M to 9.3×10^{-6} M (Figure S2(1)). By applying Lambert-Beer law, the molar extinction coefficient of complex **2** was calculated as $\varepsilon_{(375)} = 5000\ M^{-1}cm^{-1}$, and $\varepsilon_{(260)} = 72000\ M^{-1}cm^{-1}$ (Figure S2 (2)(3)). The molar extinction coefficient of AS1411 was obtained as $\varepsilon_{(260)} = 368800\ M^{-1}cm^{-1}$.[3]

From Figure S2 (4), the **AS1411-2** has exhibited combined absorption features from both complex **2** and AS14111 components. Specifically, the intensive absorption at 260 nm was majorly contributed by AS1411, and the absorption at 375 nm was primarily attributed to complex **2**. In order to calculate the relative molecular ratio of complex **2** and AS1411 in the **AS1411-2** conjugate, the absorbance at 260 nm (AS411) and 375 nm (complex **2**) were measured, and the absorption contribution from complex **2** at 260 nm and the contribution from AS1411 at 375 nm were corrected (equation (1)). The concentrations of complex **2** and AS1411 aptamer in the conjugate were calculated using the following equations (1), (2), and (3). (*A* is the observed absorbance of **AS1411-2** conjugate at noted wavelength). The molecular ratio of complex **2** and AS1411 in the **AS1411-2** conjugate was estimated by average of three measurements.

(1) $A*_{375} = A_{375} - A_{375(\mathrm{AS1411})}$

(2) $\mathrm{NHC\text{-}Au(M)} = \dfrac{A*_{375}}{\varepsilon_{375}}$

(3) $\mathrm{AS1411(M)} = \dfrac{A_{260} - (A*_{375} \times \varepsilon_{260}/\varepsilon_{375})}{368800}$

ε_{375}= Molar extinction coefficient of NHC-Au **2** at 375 nm
ε_{260}= Molar extinction coefficient of NHC-Au **2** at 260 nm

Sample	Concentration of AS1411 ($\times 10^{-6}$M)	Concentration of NHC-Au 2 ($\times 10^{-6}$M)	NHC-Au 2(M)/AS1411(M)
1	3.71	3.60	0.97
2	4.35	5.00	1.15
3	5.32	5.80	1.09

Table S 2. The calculated concentration of AS1411 and NHC-Au **2** in **AS1411-2**, and the drug/aptamer ratio calculated from UV-visible absorption experiments. The average drug/aptamer ratio from three independent experiment is calculated as 1.07±0.09.

11. NMR sprctra

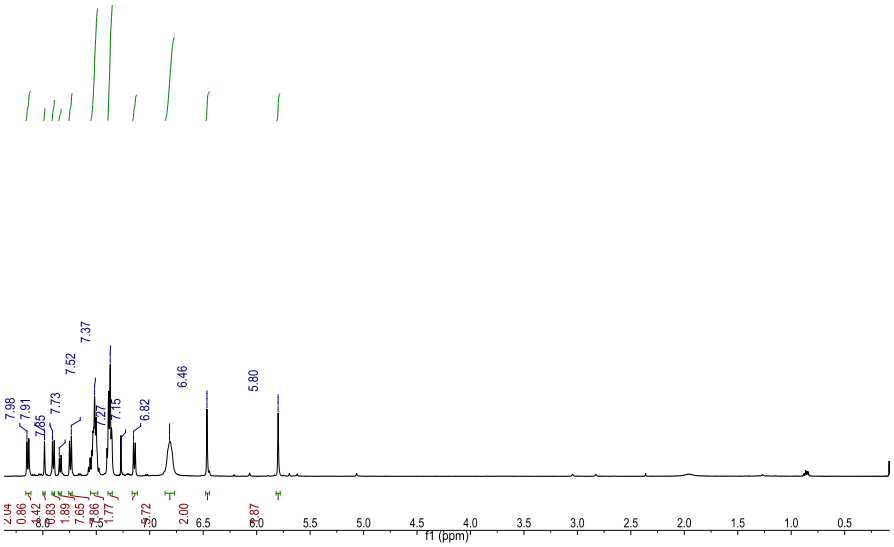

Figure S 2. ^{1}H NMR spectrum of **2** in $CDCl_3$.

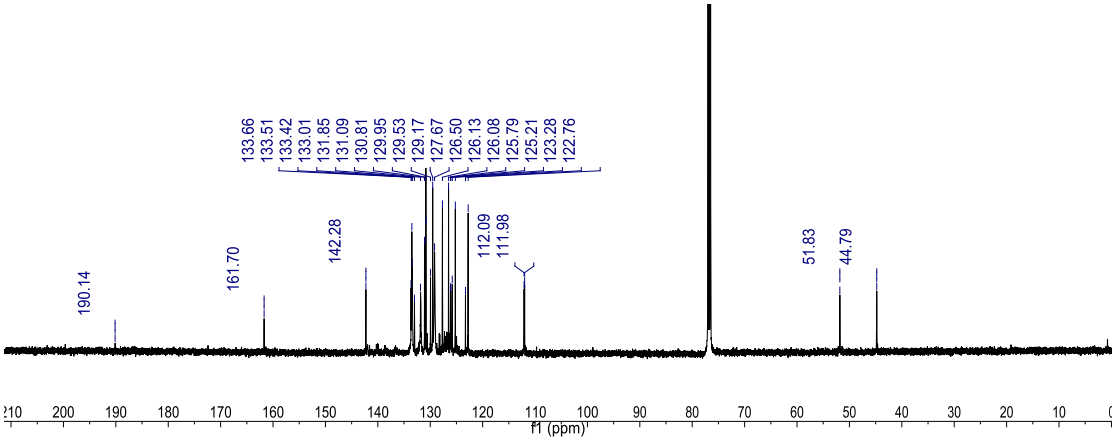

Figure S 3. ^{13}C NMR spectrum of **2** in $CDCl_3$

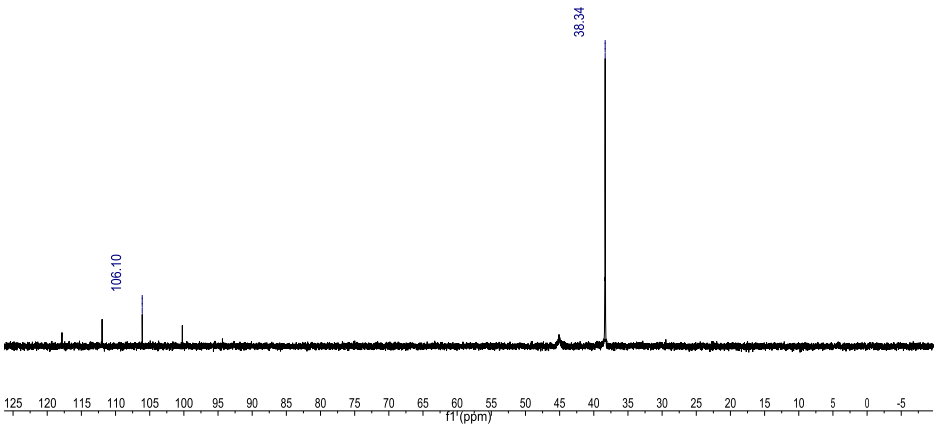

Figure S 4. ^{31}P NMR spectrum of **2** in $CDCl_3$.

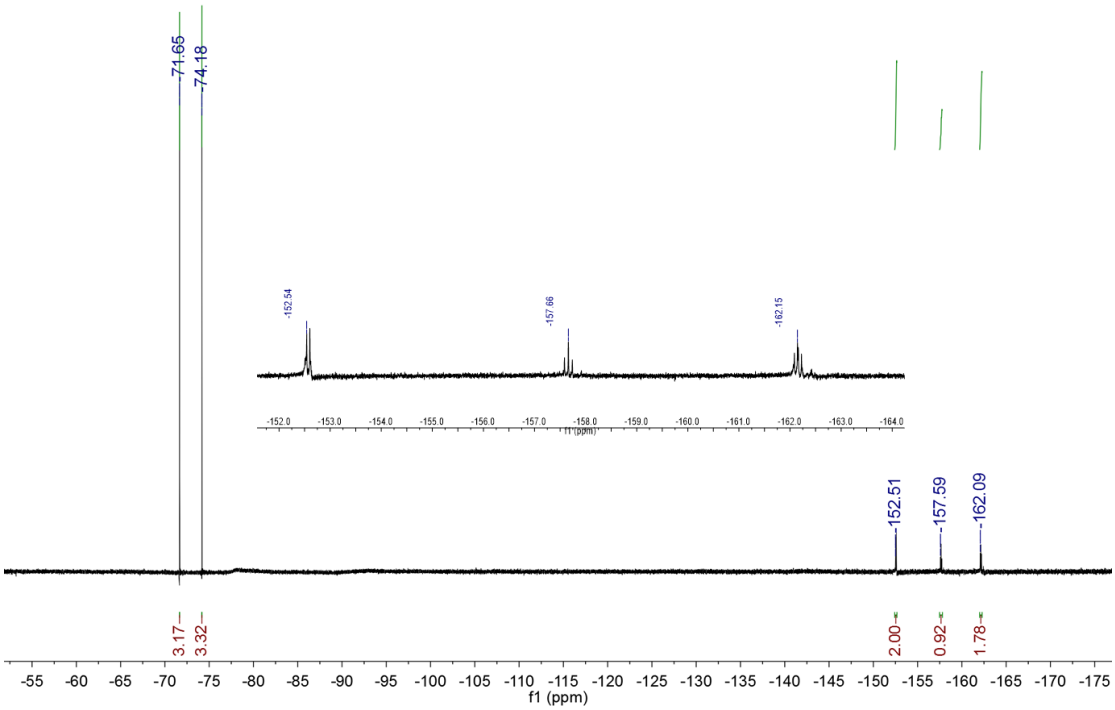

Figure S 5. ^{19}F NMR spectrum of **2** in $CDCl_3$.

12. MTS data

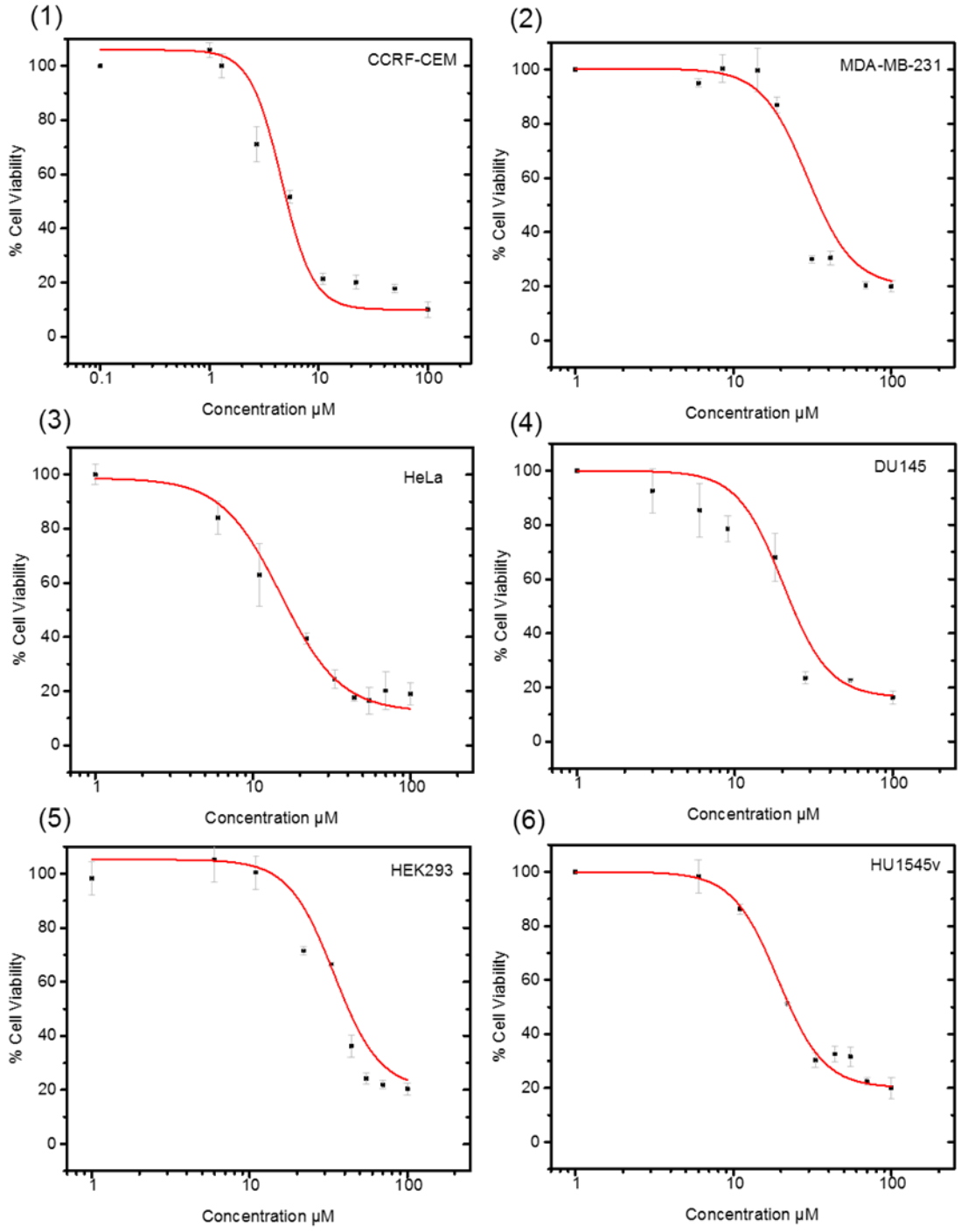

Figure S 6. MTS assay of NHC-Au complex **2** to (1) CCRF-CEM cells; (2) MDA-MB-231 cells; (3) DU145 cells; (4) Hela cells; (5) HEK293 cells; (6) HU1545v cells.

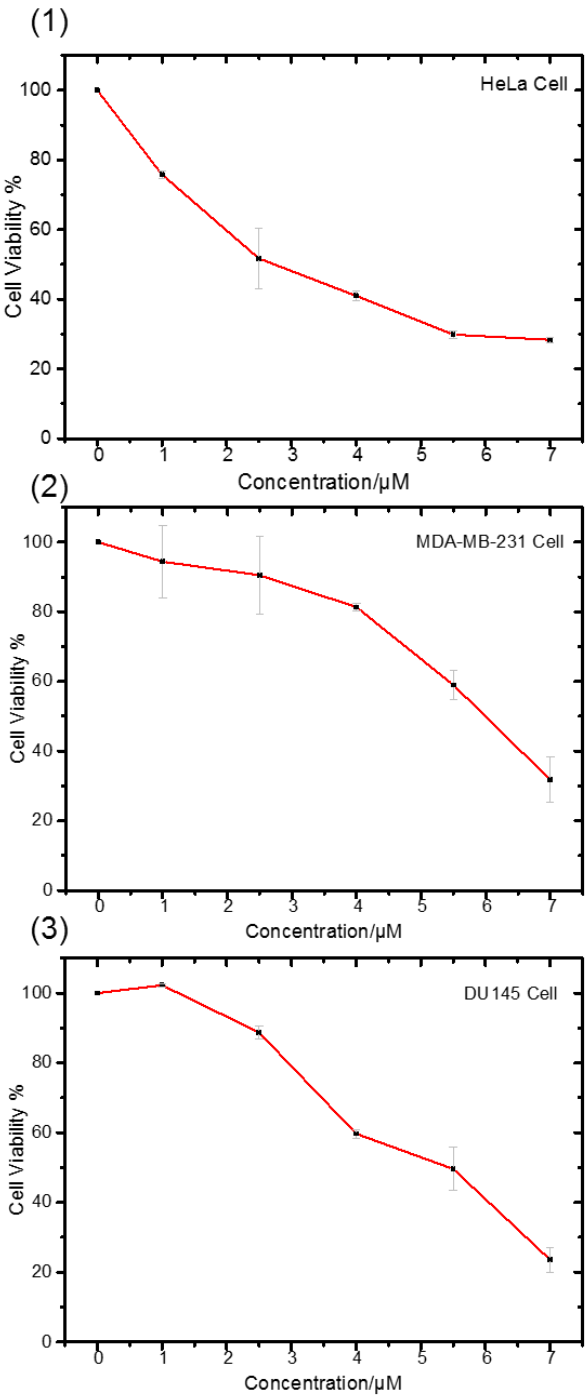

Figure S 7. MTS assay of **AS1411-2** conjugate towards (1) Hela cell; (2) MDA-MB-231 cell; (3) DU145 cell.

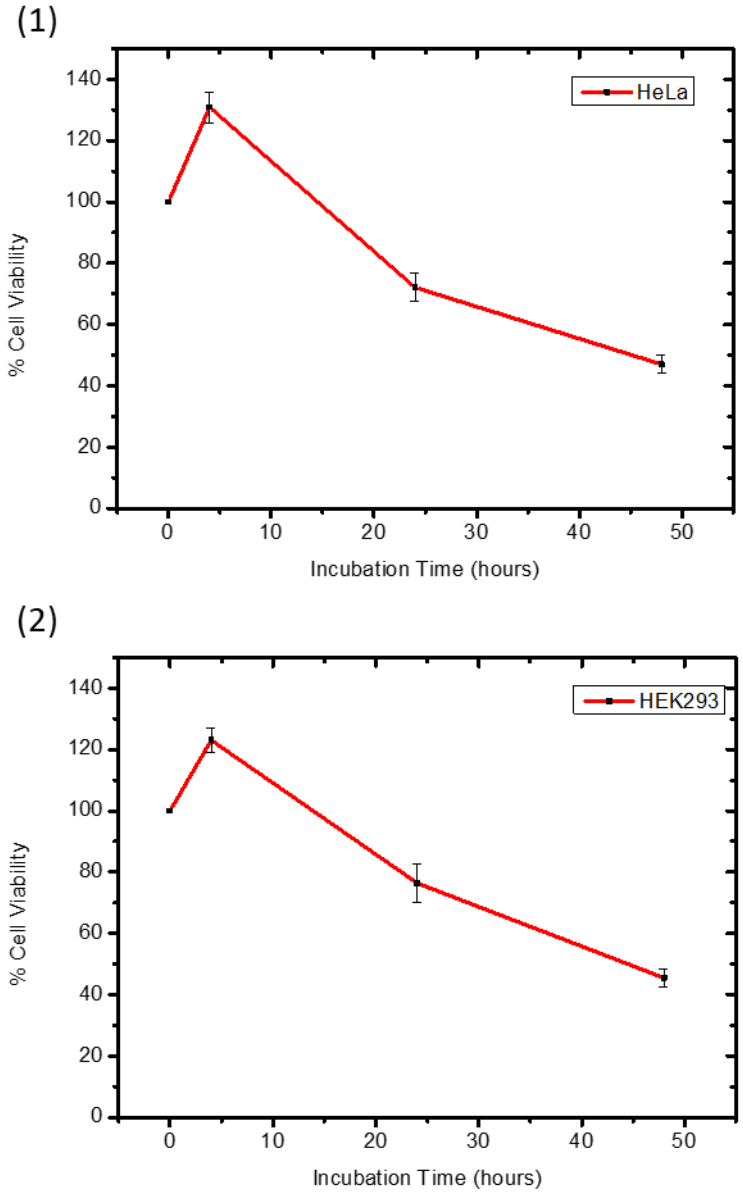

Figure S 8. Cell viability of (1) HeLa and (2) HEK293 cells after incubate with complex **2** at 4 µM for 4 hours, 24 hours and 48 hours.

13. Confocal images

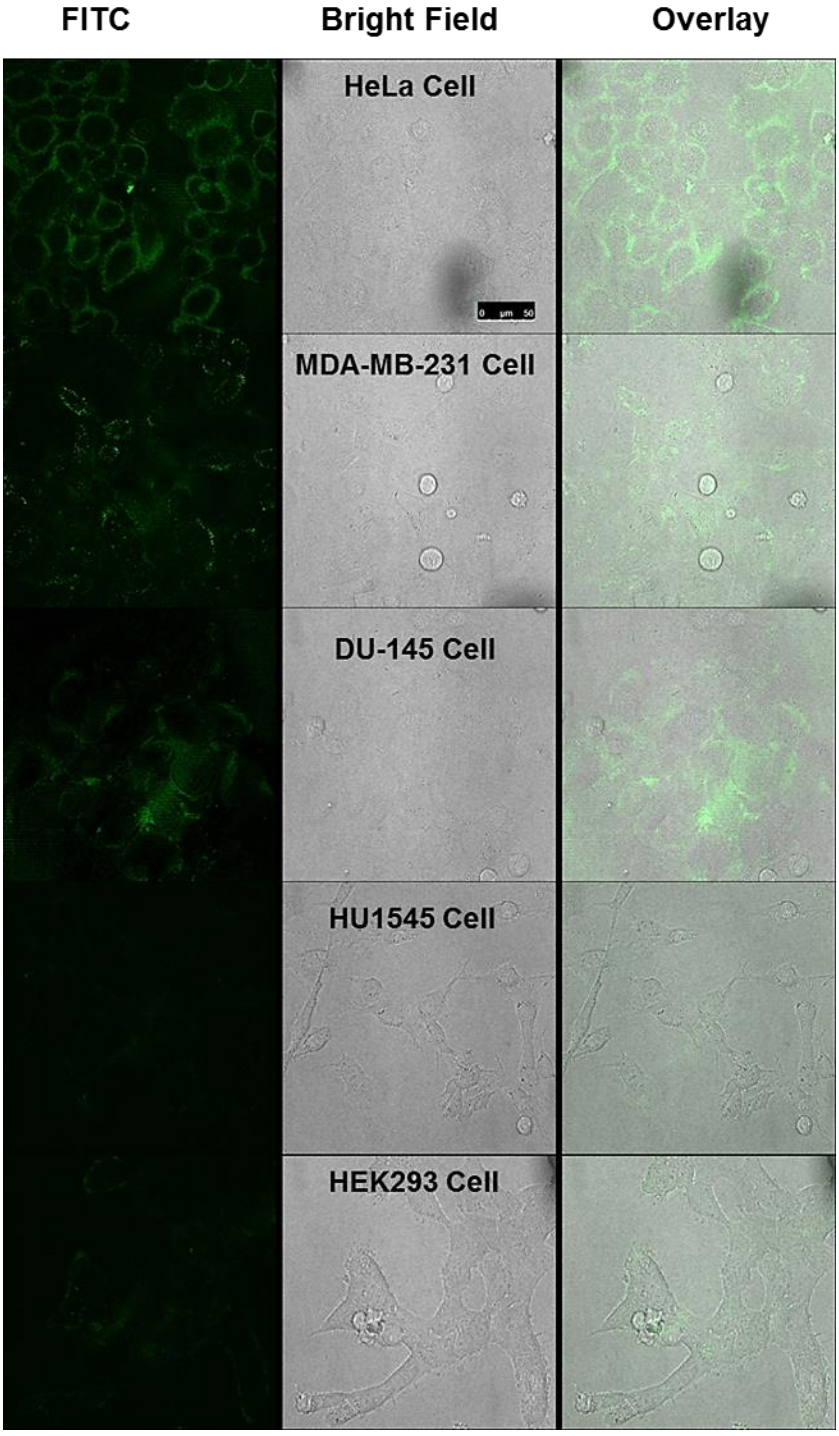

Figure S 9. Confocal laser scanning microscopy images of Hela cell, MDA-MB-231 cell, DU-145 cell, HU1545 cell and HEK293 cell treated with 100 nM AS1411-**2** conjugate. Left panels are FITC fluorescence, the middle panels are the cell in bright field, and right panels are the overlay of FITC fluorescence and the bright-field image. Scale bars=50 μm.

14. Gel electrophoresis image

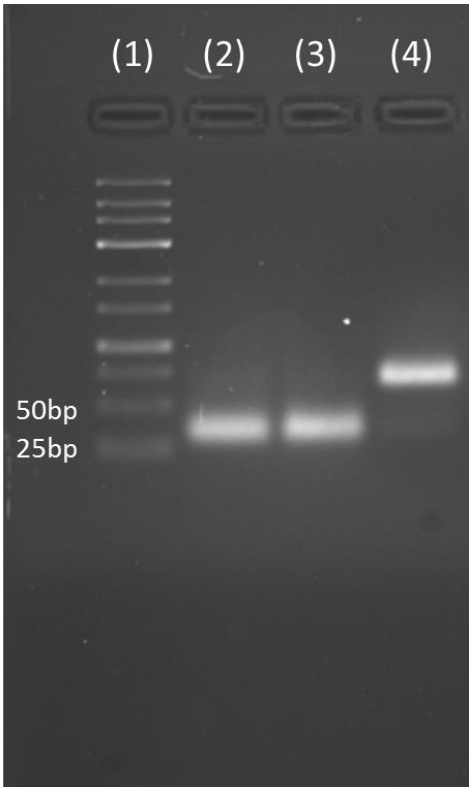

Figure S 10. Agarose gel electrophoresis of DNA marker (lane 1), AS1411 (lane 2); AS1411-**2** conjugate (lane 3); the 20-min component from HPLC separation (lane 4).

15. In vivo viability of different cell lines after treatment with AS1411-1 conjugate.

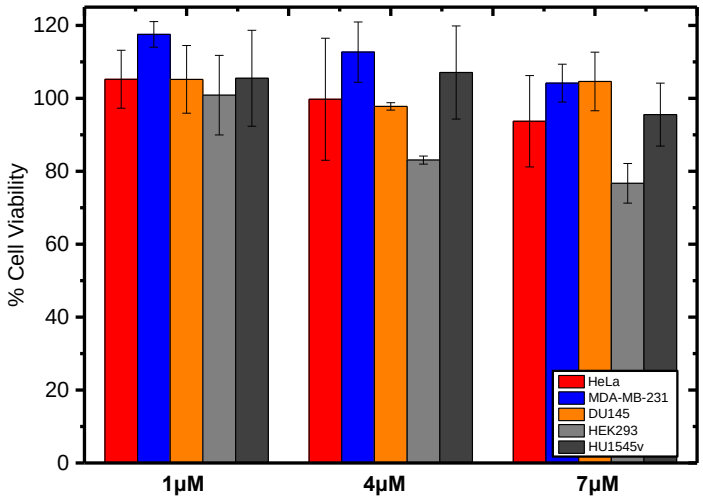

Figure S 11. In vivo viability of different cell lines after treatment with AS1411-**1**. **conjugate.**

References

1. Z. Xiao, D. Shangguan, Z. Cao, X. Fang and W. Tan, *Chemistry-a European Journal*, 2008, **14**, 1769-1775.
2. L. Q. Zhang, Z. Y. Yang, K. Sefah, K. M. Bradley, S. Hoshika, M. J. Kim, H. J. Kim, G. Z. Zhu, E. Jimenez, S. Cansiz, I. T. Teng, C. Champanhac, C. McLendon, C. Liu, W. Zhang, D. L. Gerloff, Z. Huang, W. H. Tan and S. A. Benner, *Journal of the American Chemical Society*, 2015, **137**, 6734-6737.
3. The extinction coefficient of the aptamer was calculated at https://www.idtdna.com/site