For submission to *Chem. Commun.*

Electronic Supplementary Information

for

Correlated Mass Spectrometry and Confocal Microscopy Imaging Verifies Dual-Targeting Action of an Organoruthenium Anticancer Complex

Suyan Liu,[a,b] Wei Zheng,[a] Kui Wu,[a,*] Yu Lin,[a] Feifei Jia,[a] Yang Zhang,[a] Zhaoying Wang,[a] Qun Luo,[a] Yao Zhao,[a] Fuyi Wang [a,*]

a. Beijing National Laboratory for Molecular Sciences; National Centre for Mass Spectrometry in Beijing; CAS Key Laboratory of Analytical Chemistry for Living Biosystems, Institute of Chemistry, Chinese Academy of Sciences, Beijing 100190, P. R. China

b. University of Chinese Academy of Sciences, Beijing, 100049, P. R. China

Corresponding Emails: fuyi.wang@iccas.ac.cn; wukui@iccas.ac.cn.

Table of contents

The videos of depth profiling of MCF-7 cells shown in Figure S5 are provided in separated files.

Experimental section

Materials .

Organometallic ruthenium(II) complexes [(η^6-benzene)Ru(*N*,*N*-L)Cl]PF_6 (**1**[PF_6], L = 4-(3′-chloro-4′-fluoroanilino)-6-(3-(2-aminoethyl)amino-propoxy)-7-meth-oxyquinazoline) and [(η^6-benzene)Ru(en)Cl]PF_6 (**2**[PF_6], en = ethylenediamine) were synthesized according to procedures reported previously (R. E. Morris, R. E. Aird, P. del Socorro Murdoch, H. Chen, J. Cummings, N. D. Hughes, S. Parsons, A. Parkin, G. Boyd, D. I. Jodrell, P. J. Sadler, *J. Med. Chem.* **2001**, *44*, 3616-3621; W. Zheng, Q. Luo, Y. Lin, Y. Zhao, X. Wang, Z. Du, X. Hao, Y. Yu, S. Lu, L. Ji, X. Li, L. Yang, F. Wang, *Chem. Commun.* **2013**, *49*, 10224-10226). Briefly, the ruthenium dimer [(η^6-benzene)$RuCl_2$]$_2$ reacted in methanol with 4-(3′-chloro-4′-fluoroanilino)-6-(3-(2-aminoethyl)aminopropoxy)-7- methoxyquinazoline (S. Lü, W. Zheng, L. Y. Ji, Q. Luo, X. Hao, X. C. Li, F. Y. Wang, *Eur. J. Med. Chem.* **2013**, *61*, 84-94) or ethylenediamine (AR grade from Beijing Xingjin Chemicals Co., China) at 298 K for 4 h. Then ammonium hexafluorophosphate (NH_4PF_6; AR grade from Shanghai Darui Finechemical Co., Ltd., Shanghai, China) was added to the mixture and further stirred for 10 min. The solution was slowly condensed and stood at room temperature for precipitation. The precipitates were collected by filtrating and washing with methanol and ether in succession to give the respective product.

Cisplatin was obtained from Sigma (USA) and other common reagents like ammonium acetate, EDTA and HCl used here are all obtained from commercially available sources and were used as supplied. The Ru standard and HNO_3 (BV−III

grade) for ICP-MS analysis were obtained from NCS Analytica Instruments and BICR (Beijing, China), respectively. The deionized water used in the experiments was prepared by a Milli-Q system (Millipore, Milford, MA).

The human breast cancer cell line MCF-7 and the human cervical cancer cell line HeLa were obtained from the Center for Cell Resource of Peking Union Medical College Hospital (Beijing, China). Cell nucleus staining fluorescent probe Hoescht33342 and cell membrane staining fluorescent probe DiI were purchased from Sigma (USA) and Beyotime Institute of Biotechnology (Shanghai, China), respectively. Nucleon genomic DNA extraction kit and BCA protein assay kit were bought from Tiangen Biotech (Beijing, China), membrane protein extraction kit from BestBio (Shanghai, China) and EGF from Sigma (USA). 90% high glucose DMEM with 10% fetal bovine serum and 1% Penicillin-Streptomycin were obtained from Invitrogen (USA), and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) from Sigma. The 96-well plates were bought from LunaTM Automated Counter (Logos Biosystems, Korea). Anti-EGFR primary antibody, anti-tubulin primary antibody and secondary antibody were bought from Abcam Ltd. Nitrocellulose membrane was from Millipore.

Fabrication of Addressable Silicon Wafer

The polished silicon wafer was purchased from Beijing Zhongjingkeyi Technology CO., LTD (Beijing, China). The addressable silicon wafer was fabricated by photoetching method. The characteristic pattern was written by EBL Lithography

(Electron Beam Liquid, SUSS-MA6, Germany) and the patterned template was obtained through corrosion of chromium film. Then the template was exposed to UV-light to produce the photoresist mask. Inductively Coupled Plasma (ICP) (Oxford Instruments ICP180, UK) silicon etching was performed to achieve the required depth of the pattern under the protection of photoresist mask. Finally, the patterned silicon wafer was washed by acetone to remove the photoresist.

Cell Culture and Sample Preparations

The MCF-7 and HeLa cancer cells were seeded on silicon wafers at a density of 1×10^4 cells/cm^2 in a cell culture dish containing 8 mL of DMEM culture media, and incubated at 310 K under a humidified atmosphere containing 5% CO_2 to adhere overnight. The stock solution of each ruthenium complex (5 mM) in DMSO was diluted with the culture medium to a desirable concentration, and added into respective culture dish, the cells were then incubated with complex **1** or **2** for 24 h. Control cell samples were incubated alongside the drug doped cells under the same conditions. For ToF-SIMS imaging alone, after the supernatant was moved, the cells were washed three times by ammonium acetate (150 mM, pH = 7.4), then plunged into the liquid N_2 for quick freezing, and then transferred intermediately into a LGJ-12 lyophilizer (Beijing Songyuanhuaxing Technology Develop Co., Ltd) at low temperature ranging between −65 °C and −80 °C for freezing-drying overnight. For the correlated confocal fluorescence microscopy and ToF-SIMS imaging, the fluorescence dyers Hoechst33342 (1 μg mL^{-1}) and DiI (5 μM) were in turn added to

the culture dish and the cells were further incubated for 10 min and 20 min, respectively, followed by washing and lyophilization as described above.

For ICP-MS experiments, MCF-7 or HeLa cells (5×10^6 cells/10cm Petri dish) were seeded and cultured overnight, followed by incubation with 50 μM ruthenium complex **1** or **2** at 310 K for 24 h. The media was removed and the cells were washed three times with PBS (HyClone™) to remove unreacted ruthenium complexes, and redispersed in PBS containing 0.4% EDTA. After that, the cells were divided into two parts: one part was used for DNA extraction by using the Nucleon genomic DNA extraction kit and the other for membrane protein extraction by using the membrane protein extraction kit. The concentrations of extracted DNA and membrane proteins were measured by 2550 UV-vis spectrophotometer (Shimadzu, Japan) at 260 nm and BCA protein assay kit, respectively. Afterwards, the cell extracts were mixed with 0.5 mL 50% (v/v) HNO_3, sonicated for 10 min and then transferred to 20 mL conical flasks with a total of 4 mL 20% HNO_3. The conical flasks were heated on a hot plate for 30 min until the solution turned clear. Then, 4 mL of deionized water was added and the conical flasks were heated for another 30 min. When the flasks cooled down, the digests were transferred into a volumetric tube and diluted to 3 mL. The samples were stored at 277 K before ICP-MS measurement.

Confocal Laser Scanning Microscopy (CLSM)

The fluorescence imaging of cell membrane (DiI) and nuclei (Hoechst33342) were performed on FV1000-IX81 confocal laser scanning microscopy through an IX81

inverted microscope (Olympus, Tokyo, Japan). For nucleus dyer Hoechst33342, the excitation wavelength was 405 nm and emission wavelength 460 nm. For membrane dyer DiI, the excitation wavelength was 559 nm and emission wavelength 565 nm.

Time of Flight Secondary Ion Mass Spectrometry (ToF-SIMS)

ToF-SIMS analysis was carried out on ToF-SIMS V instrumentation (ION-TOF, GmbH, Munster, Germany) equipped with a pulsed Bi_3^+ liquid metal gun as a primary ion source and an O_2^+ beam as a sputtering ion source. The cell images were collected using the pulsed Bi_3^+ analysis beam by 256 × 256 pixels at ion current of 0.3 pA and the maximum ion dose density was kept below 10^{13} ions/cm^2 to avoid the sample surface damage induced by primary ion source. Dual beam depth profiling used a 30 keV pulsed Bi_3^+ liquid metal gun (ion current = 0.3 pA) for analysis and 1 keV O_2^+ beam with a current of 200 nA for sputtering in non-interlaced mode. Low energy electron gun was used for charge compensation during the analysis circles. Positive spectra were recorded and calibrated by CH^+, CH_3^+, $C_2H_3^+$, $C_3H_5^+$ and $C_5H_{15}NPO_4^+$ peaks. All the ToF-SIMS related data including spectra and images were processed by SurfaceLab 6 software ver6.3 or 6.4.

Image Processing

The Z-correction of the ToF-SIMS images was performed by Z-CorrectorGUI program available on http://www.nb.uw.edu/mvsa/software (NESAC/BIO, University of Washington) following the procedure described in the literature (M. A. Robinson,

D. J. Graham, D. G. Castner, *Anal. Chem.* **2012**, *84*, 4880-4885). Generally, the MS data (*m/z* – signal intensity) of interested peaks obtained in MCF-7 cells were exported as .bif6 files. Then all the files were imported into the ZCorrectorGUI toolbox program written in MATLAB (MathWorks, Natick, MA) to construct 2D, 3D, merged images and videos in RGB color scheme as desired. The merging of the confocal fluorescence images and ToF-SIMS images were processed by ImageJ ver. 1.49 (available at http://imagej.nih.gov/ij/).

Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

The quantitation of ruthenium binding to DNA or to membrane proteins was performed on ICP-MS 7700 (Agilent, USA) under operating conditions as follows: RF plasma source: 1350 W; Ar plasma gas: 15 L min^{-1}; Ar carrier gas: 0.8 L min^{-1}. The calibration standards of Ru (0.01, 0.1, 1, 10, 100 ppb) were newly prepared by diluting certificated standard of Ru (NCS Analytica Instruments, 1000 μg mL^{-1} in 10% HCl) with 1% HNO_3 solution.

Western Blotting

Protein samples were dissolved in a sample buffer containing 0.5 M Tris hydrochloride (pH 6.8), 20% glycerol, 2% sodium dodecyl sulphate (SDS), bromphenol blue, and dithiothreitol. The protein concentrations of cell membrane extraction were measured by Bradford method using bovine serum albumin as a standard. The proteins (40-100 μg) were separated by 8% SDS-polyacrylamide gels,

electroblotted on nitrocellulose (NC) membranes, and blocked with a solution of 5% (w/v) milk powder and 0.1% (w/v) Tween 20 in PBS (pH 7.5) for 1 h at room temperature. The NC membranes were incubated with appropriate primary antibodies for 2 h at room temperature or overnight at 4 ˚C. Then membranes were washed with PBS and incubated with a secondary antibody for 2 h. Then HRP substrate was added and the NC membranes were detected by ChemiDoc Touch system (Bio-rad). The intensities of the bands were quantitated using ImageJ.

MTT Assays

The IC_{50} values of the two ruthenium complexes inhibiting the growth of MCF-7 or HeLa cell line were measured by MTT assay with cisplatin as a positive control. The cells were seeded into a 96-well plate at a density of 4000 cells/well containing 100 μL DMEM media, and grew in the presence or absence of EGF (100 ng mL^{-1}) for 24 h. The stock solutions of complexes **1**, **2** and cisplatin in DMSO were freshly prepared (20 mM for **1** and **2**, 1 mM for cisplatin) before diluted to the desired concentration with culture media for addition to the cells, and the final concentration of DMSO in each well was kept at 0.5%. MCF-7 or HeLa cells were incubated with each tested complex at seven concentrations (200, 100, 50, 25, 12.5, 6.25 and 1 μM for complexes **1** and **2**; 100, 50, 25, 12.5, 6.25, 1 and 0.1 μM for cisplatin). After 48-h incubation, the culture medium was discarded and the cells were washed three times with PBS. Then 100 μL complete medium containing MTT (0.5 mg ml^{-1}) was added to each well for further 4 h of incubation at 310 K. After the media removed, 100 μL

of DMSO was added to dissolve the formazan crystals. Optical density (OD) value for each well was measured by a microplate reader (SpectraMax M5 Molecular Devices Corporation) at the wavelength of 570 nm. The inhibition rate (IR) was calculated based on the equation as followings:

$$IR\ (\%) = [1 - (OD_{compound} - OD_{blank}) / (OD_{control} - OD_{blank})\ ] \times 100\%.$$

All reported IR values were averages of three independent experiments and expressed as mean ± SD (standard deviation).

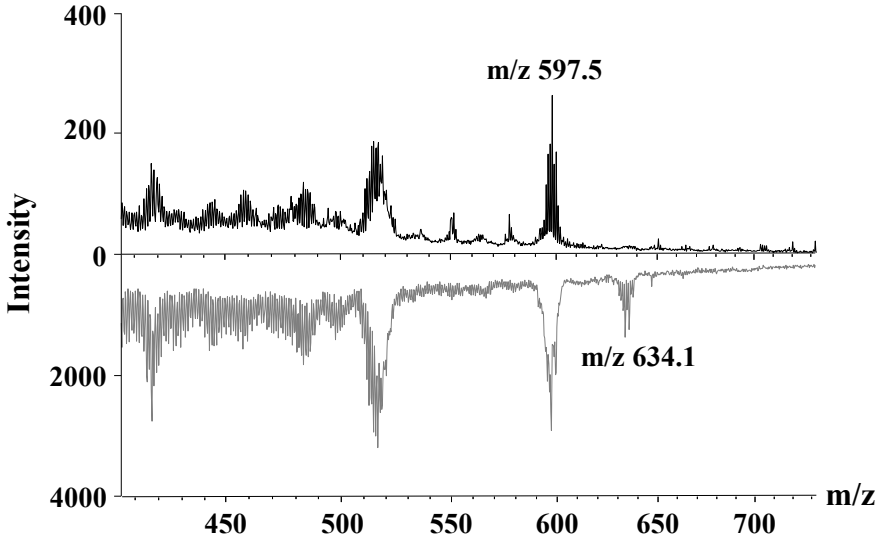

Figure S1. Regions of the mass spectra of complex **1** obtained by ToF-SIMS under mass spectrometric mode from a dried solution sample (grey) and a freeze-dried MCF-7 cell sample (black), showing the Ru-containing peaks including the molecular ions at *m/z* 634.1 (**1**; the most abundant isotopomer, the same hereinafter) and the fragment ions of the hydrolytic adduct at *m/z* 597.5 [**1** – Cl – H]$^+$. The mass spectra for the dried solution sample and the cell sample are the sum of 11 scans and 6 scans, respectively, on the sample surface.

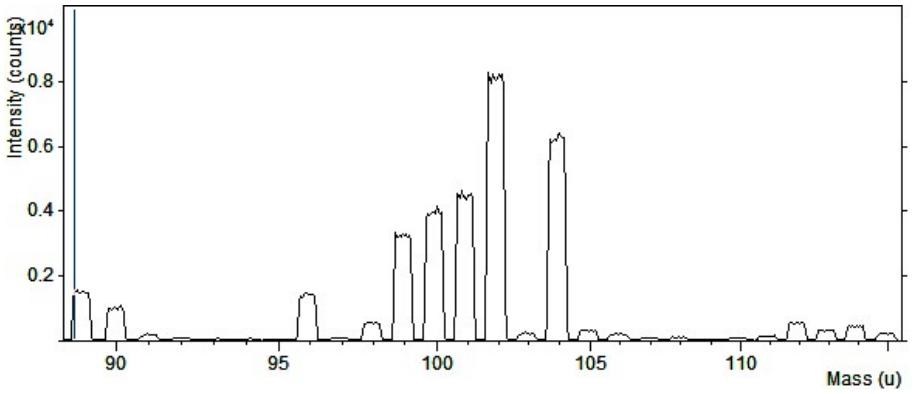

Figure S2. Region of the mass spectrum obtained by ToF-SIMS under imaging mode from freeze-dried MCF-7 cells treated with complex **1**, showing the Ru^+ isotopic peaks arising from fragmentation of complex **1** over the entire sputter depths. It is notable that the ToF-SIMS analysis has only unit mass resolution under imaging mode, thus the naked Ru ions were detected at *m/z* 96, 98 (in the lowest abundance), 99, 100, 101, 102 and 104 and the imaging of Ru^+ shown in Figures 2, 4, S3 and S9 was recorded by collecting the signals of Ru ions at *m/z* 96, 99, 100, 101, 102 and 104.

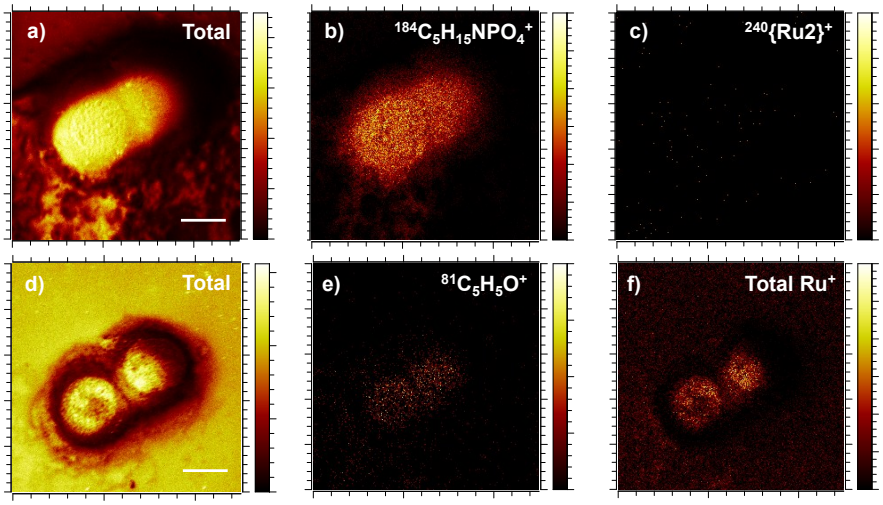

Figure S3. ToF-SIMS imaging of MCF-7 cells treated with complex **2**. (a – c) Ion images of cell surface; (d – f) Overlaid images over the 1st – 300th slices of the sputtering depths. Scale bar: 20 μm. The ions at *m/z* 184 and 81 arising from fragmentation of phosphocholine and deoxyribose provide subcellular markers for membrane and nucleus, respectively. Ru-containing fragment {Ru2}$^+$ at *m/z* 240 ($C_8H_{14}N_2Ru^+$) corresponds to the hydrolytic adduct, [**2** – Cl – H]$^+$, of complex **2**, and the Ru^+ ions, including $^{96}Ru^+$, $^{99}Ru^+$, $^{100}Ru^+$, $^{101}Ru^+$, $^{102}Ru^+$ and $^{104}Ru^+$, were produced by fragmentation of complex **2** during O_2^+ beam sputtering.

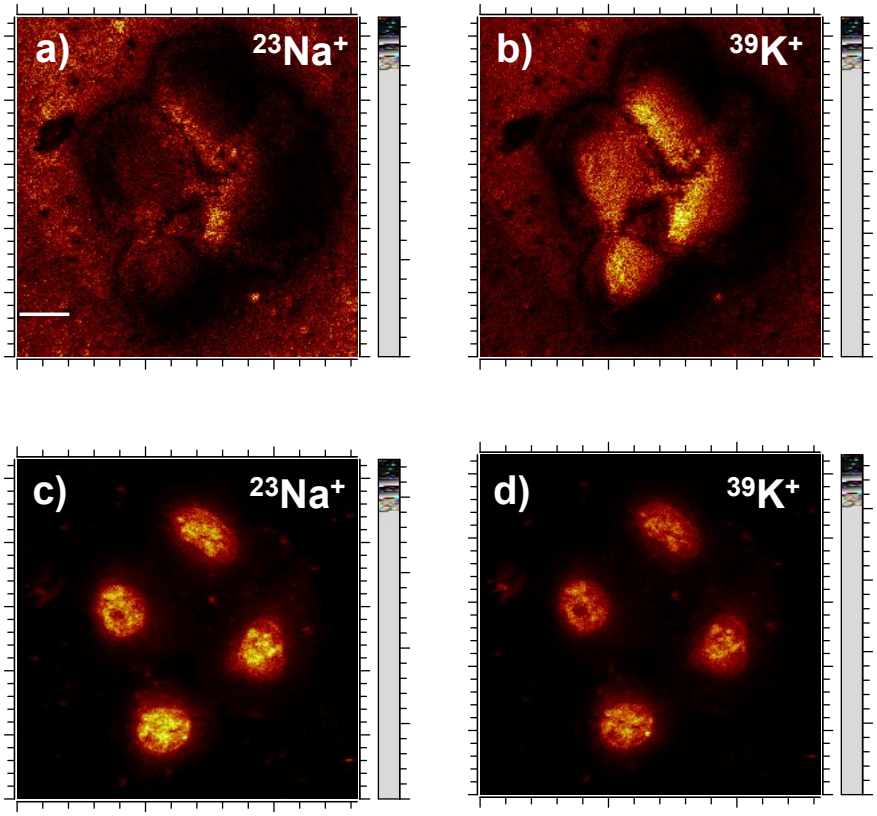

Figure S4. ToF-SIMS imaging of $^{23}Na^+$ and $^{39}K^+$ in MCF-7 cells treated with complex **1**. (a, b) Ion images of cell surface; (c, d) Overlaid images over the 51st – 650th slices of the sputtering depths. Scale bar: 20 μm.

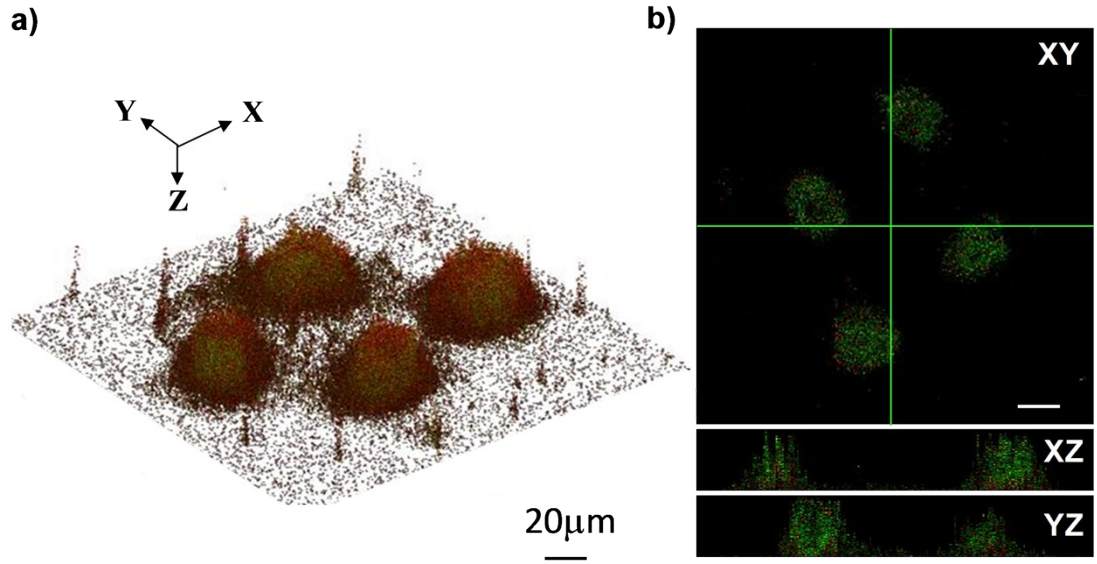

Figure S5. Depth profile of MCF-7 cells exposed to complex **1**. (a) 3D image constructed by images over the 51^{th} – 650^{th} slices of the sputtering depths, and (b) snapshot images from different cross-sections. Green: K^+ ions; Red: Ru^+; Scale bar: 20 μm. Note: The correction of Z axis was performed for the 3D rendering of the cells by using Z-CorrectorGUI program (http://www.nb.uw.edu/mvsa/software), but the z axis does not represent the real thickness of the cells. The videos corresponding to the depth profiling are also provided as separated files.

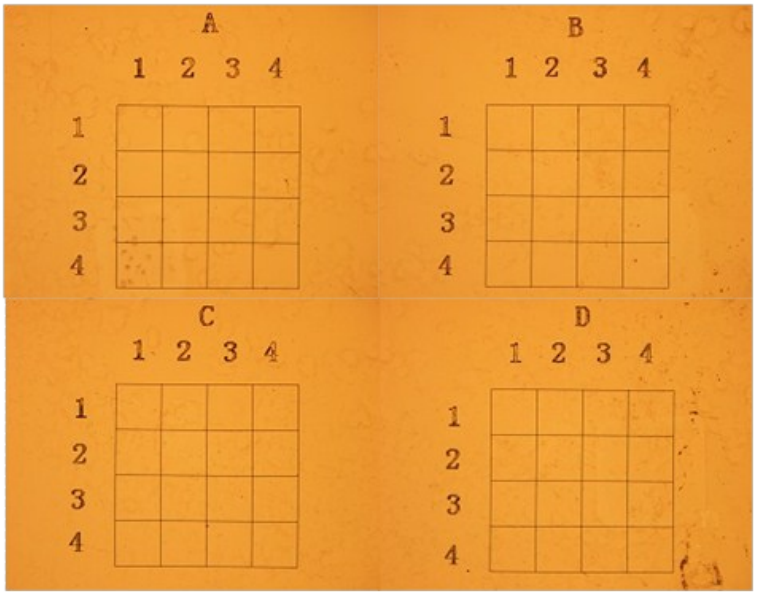

Figure S6. Addressable silicon wafer patterned with square matrix, each grid is 200 × 200 μm^2.

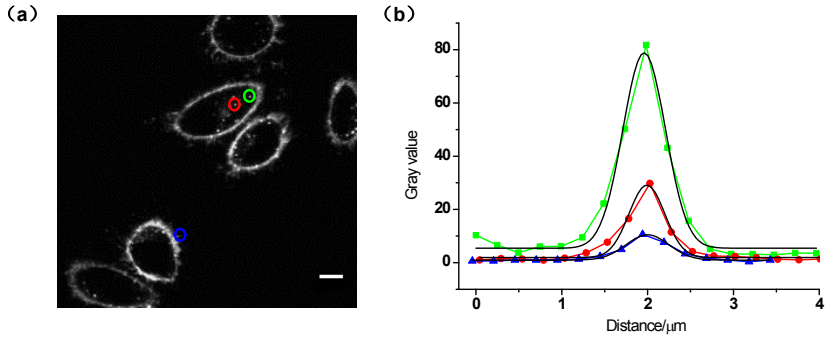

Figure S7. To determine the lateral resolution of confocal laser microscopic fluorescence imaging, line scans were performed in freeze-dried MCF-7 cells stained with cell membrane probe DiI (scale bar: 10 μm). Line scans were drawn through the cell body or cilium region shown in colored circles and Gaussian fits (black) were also applied to give an average full width at half maximum (FWHM) of 548 ± 70 nm.

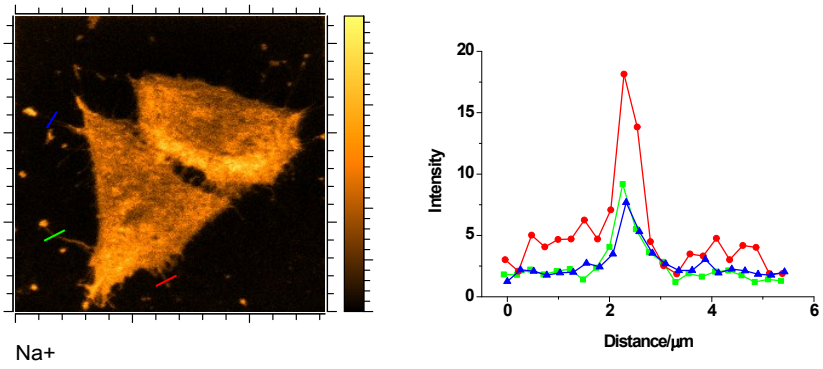

Figure S8. To determine the lateral resolution of ToF-SIMS imaging, line scans were performed in freeze-dried MCF-7 cells. Line scans were drawn through the cell cilium region shown from the $^{23}Na^{+}$ image indicated by colored lines. Applying the 16–84% criterion (C. Lechene, F. Hillion, G. McMahon, D. Benson, A. M. Kleinfeld, J. P. Kampf, D. Distel, Y. Luyten, J. Bonventre, D. Hentschel, K. M. Park, S. Ito, M. Schwartz, G. Benichou, G. Slodzian, *J. Biol.* **2006**, *5*, 20), instead of the FWHM, the average lateral resolution of three line scans was calculated to be 262 ± 31 nm.

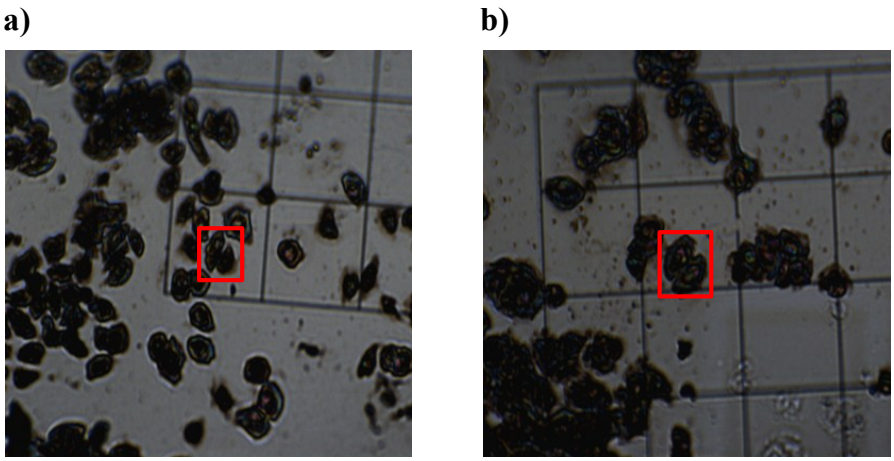

Figure S9. Reflected optical images taken by micro-camera built in the ToF-SIMS main chamber of lyophilized MCF-7 cancer cells treated with complex **1** (a) and complex **2** (b) on addressable silicon wafers. The cells framed in red were selected for the co-localization imaging and the results are shown in Figure 4 and Figure S9, respectively.

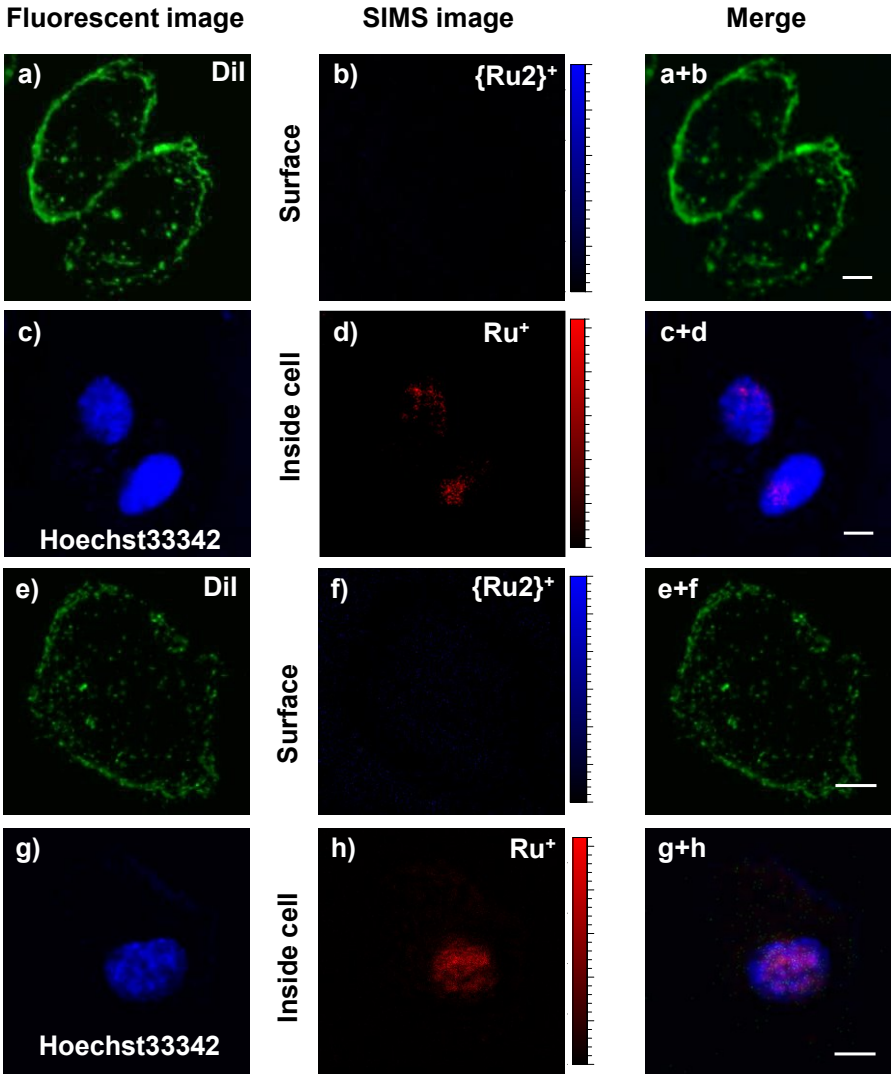

Figure S10. Combined TOF-SIMS and Confocal fluorescence microscopy images of MCF-7 (a-d) and HeLa (e-h) cancer cells exposed to 100 μM complex **2**. The Ru-containing fragments $\{Ru2\}^+$ (m/z 240, $C_8H_{10}N_2Ru$) was the hydrolytic adduct of complex **2** and the Ru^+ ions were the sum of $^{96}Ru^+$, $^{99}Ru^+$, $^{100}Ru^+$, $^{101}Ru^+$, $^{102}Ru^+$ and $^{104}Ru^+$ isotopes produced by fragmentation of complex **2** during sputtering. Scale bar: 15 μm.

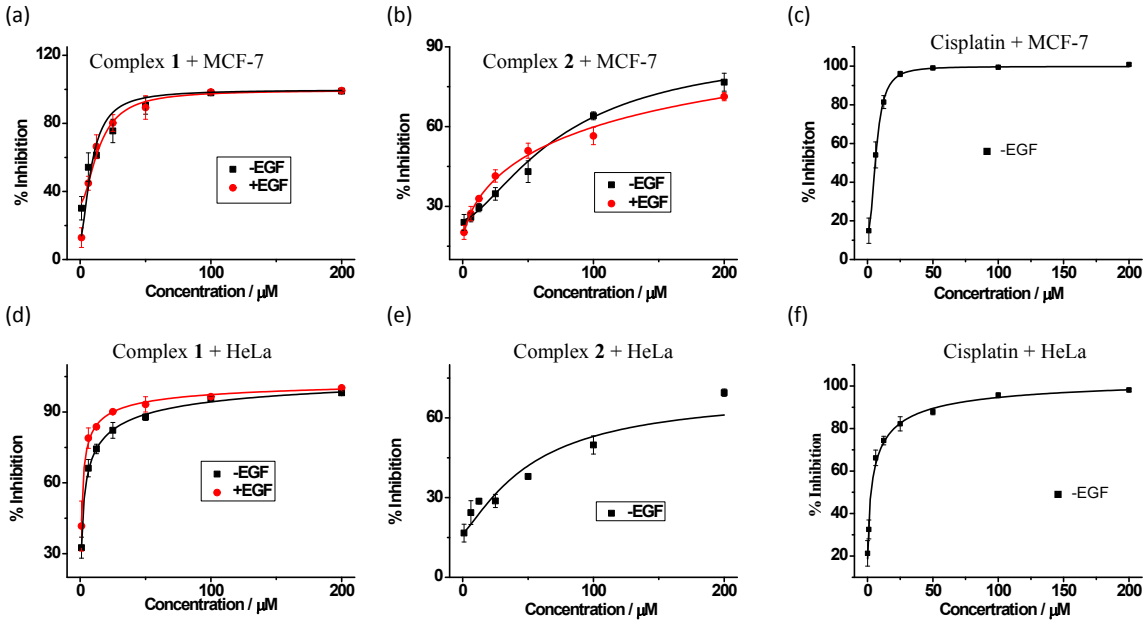

Figure S11. Dose dependent antiproliferation curves of complexes **1** and **2** against MCF-7 and HeLa cancer cell lines in the presence or absence of exogenous EGF (100 ng/mL). Cisplatin was used as a positive control in the test of antiproliferative activity. The IC_{50} of complex **2** to HeLa cell line was higher than 100 μM in the presence of exogenous EGF, and the corresponding antiproliferation curve is not shown here. It is of worth to point that HeLa cancer cells overexpress EGFR at much higher level than MCF-7 cancer cells, leading to largely decrease in the antiproliferative activity of complex **2** against HeLa cell lines in the presence of exogenous EGF because complex **2** is not active towards EGFR.

Table S1. IC_{50} for inhibition of the growth of MCF-7 and HeLa cancer cells by ruthenium complexes **1** and **2**. The cells were exposed to each tested compound for 48 h in the presence or absence of EGF (100 ng/mL).

	MCF-7 (μM)		HeLa (μM)	
	-EGF	+EGF	-EGF	+EGF
Complex **1**	14.7±1.98	9.9±1.95	4.6±1.62	1.36±0.34
Complex **2**	48.57±3.37	43.95±3.54	63.50±2.92	>100
Cisplatin	7.00±1.16	N.A.	13.86±1.85	N.A.

N.A.: Not analyzed.