Electronic Supplementary Information (ESI)

A dual cytotoxic and anti-angiogenic water-soluble gold(III) complex induces endoplasmic reticulum damage in HeLa cells

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Contents

1. General experimental procedure
   A. Materials
   B. Instruments
   C. Synthesis
   D. Cell lines and cell cultures
   E. Cellular-uptake experiment
   F. Transmission electron microscopy
   G. Cell transfection for vacuoles formation observation
   H. RNA extraction
   I. Oligonucleotide microarray analysis
   J. Reverse transcriptase-polymerase chain reaction (RT-PCR)
   K. Western blotting analysis
   L. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay
   M. Hoechst 33342 nuclear staining experiment
   N. Cell-cycle analysis
   O. Tube-formation assay

2. Figures and tables

3. References
1. General experimental procedure

**A. Materials.** All chemicals were purchased from Sigma-Aldrich, unless otherwise noted. Biguanidemonohydrochloride was purchased from Shanghai Rory Fine Chemical Co., Ltd. Biuret was obtained from Fisher Scientific. The solvents used for synthesis were of analytical or HPLC grade.

**B. Instrumentation.** $^1$H-NMR spectra were recorded on a Bruker AVANCE 400 Fourier-Transform NMR spectrometer (chemical shift in ppm). 2-D H-H COSY NMR spectra were recorded on a Bruker AVANCE 600 Fourier-transform NMR spectrometer. Positive-ion FAB (Fast Atom Bombardment) mass spectra were recorded on a Finnigan MAT95 mass spectrometer. Negative-ion ESI (electrospray ionization) mass spectra were recorded on a Finnigan LCQ mass spectrometer. Elemental analyses were conducted in the Institute of Chemistry, The Chinese Academy of Sciences, Beijing. UV-Vis absorption spectra were recorded on a Perkin-Elmer Lambda 900 UV-Visible spectrophotometer. The transmission electron micrographs were taken under a 208S Philips transmission electron microscopy (TEM). Optical and fluorescence micrographs were taken on a Zeiss Axiovert 200M inverted fluorescence microscopy. The morphology of cells was taken by a Nikon Coolpix 5000 camera.

**C. Synthesis**

2-(4-$n$-Butylphenyl)pyridine.$^1$ A mixture of 1-bromo-4-$n$-butylbenzene (1.00 g, 4.692 mmol), 2-(tributylstannyl)pyridine (2.33 g, 6.335 mmol), Pd(PPh$_3$)$_2$Cl$_2$ (263.46 mg, 0.375 mmol) and LiCl (994 mg, 23.46 mmol) in anhydrous toluene was degassed for about 10 min, and then heated to reflux for 20 h under nitrogen atmosphere. After cooling to the room temperature, a saturated KF solution (10 mL) was added, and the mixture was stirred for another 30 min, followed by filtration.
CH₂Cl₂ (200 mL) and 5% NaHCO₃ (150 mL) were added to the filtrate. The organic phase was separated and dried with Na₂SO₄, after which the solvent was evaporated. The crude product was purified by chromatography (silica gel; hexanes-ether, 30:1) to give the final pure product as yellow oil. Yield: 0.685 g, 69.1%. ¹H-NMR (400 MHz, CD₃OD): δ = 8.50 (d, J = 4.8 Hz, 1H), 7.85 (m, 4H), 7.32 (m, 3H), 2.67 (t, J = 7.7 Hz, 2H), 1.64 (m, 2H), 1.38 (m, 2H), 0.95 (t, J = 7.4 Hz, 3H). ¹³C-NMR (100 MHz, CD₃OD): δ = 150.16, 138.90, 129.96, 128.09, 122.43, 36.37, 34.84, 23.37, 14.29. FAB-MS (+ve, m/z): 212.2 [M]⁺.

[Au³⁺(2-(4-n-butylyphenyl)pyridyl)Cl₂]⁺ To a stirring solution of K[AuCl₄] (297.6 mg, 0.792 mmol) in distilled water (20 mL) was added dropwise 2-(4-n-butylyphenyl)pyridine (183.8 mg, 0.87 mmol) in acetonitrile (2.5 mL). After stirring overnight, the resulting yellow precipitate was filtered and washed with water and hexane. The dried yellow solid was then heated at 443–453 K until all the solid changed to the final product. Yield: 152 mg, 40.2%. ¹H-NMR (300 MHz, DMSO-d₆): δ = 9.49 (d, J = 6.0 Hz, 1H), 8.35 (d, J = 4.1 Hz, 2H), 7.87 (d, J = 7.8 Hz, 1H), 7.72 (m, 1H), 7.64 (s, 1H), 7.32 (d, J = 7.5 Hz, 1H), 2.67 (t, J = 7.5 Hz, 2H), 1.57 (m, 2H), 1.33 (m, 2H), 0.91 (t, J = 7.3 Hz, 3H). FAB-MS (+ve, m/z): 442.1 [M-Cl]⁺. Anal. Calcd for C₁₅H₁₆AuCl₂N: C 37.68, H 3.37, N 2.93. Found: C 37.73, H 3.49, N 2.89.

[Au³⁺(2-(4-n-butylyphenyl)pyridyl)biguanide]Cl (1). To [Au³⁺(2-(4-n-butylyphenyl)pyridyl)Cl₂] (30.0 mg, 0.0629 mmol) was added biguanidemonohydrochloride (17.7 mg, 0.129 mmol) and tert-butylibutoxide (29.7 mg, 0.265 mmol) in methanol (3.5 mL), and the mixture was stirred at room temperature for 3 h. Then, the mixture was filtered and washed with hot methanol (343 K) and water. Yield: 19 mg, 55.6%. ¹H-NMR (400 MHz, DMSO-d₆): δ = 9.03
(d, $J = 5.5$ Hz, 1H), 8.36 (m, 2H), 7.95 (m, 1H), 7.68 (t, $J = 5.0$ Hz, 1H), 7.60 (s, 1H), 7.31 (d, $J = 7.5$ Hz, 1H), 7.19 (s, 1H), 6.65 (s, 2H), 6.30 (s, 2H), 6.11 (s, 1H), 2.71 (t, $J = 7.4$ Hz, 2H), 1.63 (m, 2H), 1.35 (m, 2H), 0.93 (t, $J = 7.2$ Hz, 3H). FAB-MS (+ve, $m/z$): 507.2 [M$^+$. Anal. Calcd for $C_{17}H_{22}AuClN_6\cdot0.5H_2O$: C 37.00, H 4.20, N 15.23. Found: C 37.12, H 4.14, N 15.24.

$[\text{Au}^{III}(\text{2-phenylpyridyl)biguanide}]Cl$ (2). To $[\text{Au}^{III}(\text{2-phenylpyridyl)Cl}_2]$ (40.0 mg, 0.095 mmol) was added biguanidemonohydrochloride (26.04 mg, 0.190 mmol) and tert-butylbutoxide (42.58 mg, 0.380 mmol) in methanol (4.5 mL), and the mixture was stirred overnight at room temperature. Then, the mixture was filtered and washed with hot methanol (343 K) and water. Yield: 44.0 mg, 95.3%. $^1$H-NMR (400 MHz, DMSO-$d_6$): $\delta = 8.99$ (d, $J = 5.8$ Hz, 1H), 8.43 (m, 2H), 8.08 (dd, $J = 2.5$ Hz, $J = 6.7$ Hz, 1H), 7.75 (m, 2H), 7.50 (m, 2H), 7.10 (s, 1H), 6.56 (s, 2H), 6.25 (s, 2H), 6.11 (s, 1H). FAB-MS (+ve, $m/z$): 451.0 [M$^+$. Anal. Calcd for $C_{13}H_{14}AuClN_6\cdotH_2O$: C 30.94, H 3.20, N 16.65. Found: C 30.78, H 2.97, N 16.58.

$[\text{Au}^{III}(\text{2-(4-n-butylphenyl)pyridyl)biuret}]$ (3). A suspension of $[\text{Au}^{III}(\text{2-(4-n-butylphenyl)pyridyl)Cl}_2]$ (40 mg, 0.0839 mmol), biuret (129.6 mg, 1.258 mmol) and potassium tert-butylbutoxide (281.8 mg, 2.515 mmol) in methanol (25 mL) was stirred at room temperature overnight. Then, the mixture was filtered and washed with hot methanol (343 K) and water. Yield: 26.0 mg, 61.0%. $^1$H-NMR (400 MHz, DMSO-$d_6$): $\delta = 9.02$ (d, $J = 4.9$ Hz, 1H), 8.31 (m, 2H), 7.89 (d, $J = 8.4$ Hz, 1H), 7.86 (s, 1H), 7.56 (m, 1H), 7.49 (s, 1H), 7.25 (d, $J = 7.1$ Hz, 1H), 5.66 (s, 1H), 5.07 (s, 1H), 2.67 (t, $J = 7.1$ Hz, 2H), 1.59 (m, 2H), 1.33 (m, 2H), 0.91 (t, $J = 6.8$ Hz, 3H). FAB-MS (+ve, $m/z$): 509.2 [M$^+$. Anal. Calcd for $C_{17}H_{19}AuN_4O_2$: C 40.17, H 3.77, N 11.02. Found: C 40.09, H 3.85, N 11.09.
[AuIII((2-phenyl)pyridyl)biuret] (4). A suspension of [AuIII((2-phenyl)pyridyl)Cl₂] (20 mg, 0.0475 mmol), biuret (73.4 mg, 0.712 mmol) and potassium tert-butyldimethylsiline (160.0 mg, 1.428 mmol) in methanol (15 mL) was stirred at room temperature overnight. Then, the mixture was filtered and washed with hot methanol (343 K) and water. Yield: 11.4 mg, 53.0%. ¹H-NMR (400 MHz, DMSO-d₆): δ = 9.08 (d, J = 5.8 Hz, 1H), 8.39 (d, J = 7.8 Hz, 1H), 8.33 (t, J = 7.8 Hz, 1H), 8.02 (d, J = 7.5 Hz, 1H), 7.93 (s, 1H), 7.63 (m, 2H), 7.42 (m, 2H), 5.72 (s, 1H), 4.94 (s, 1H). FAB-MS (+ve, m/z): 453.1 [M]+. Anal. Calcd for C₁₃H₁₁AuN₄O₂·2H₂O: C 31.98, H 3.10, N 11.48. Found: C 32.07, H 3.11, N 11.46.

D. Cell lines and cell cultures. Human cervical epithelial carcinoma (HeLa), melanoma (B16), hepatocellular carcinoma (PLC), breast carcinoma (MDA-MB-231), glioblastoma (U87), normal lung fibroblasts (CCD-19Lu) and MS1 (CRL-2279) cell lines were obtained commercially from American Type Culture Collection (ARCC, Rockville, MD, USA). Human nasopharyngeal carcinoma (SUNE1) cell line was generously provided by Prof. S. W. Tsao (Department of Anatomy, The University of Hong Kong). HeLa, B16, U87, and CCD-19Lu cells were maintained in Minimum Essential Medium (MEM, GIBCO), PLC, MDA-MB-231, and SUNE1 cells were maintained with RPMI-1640 Medium (Sigma, R4130-1L), and MS1 cells were maintained in DMEM. All the medium contained or were supplemented with 10% Fetal Bovine Serum (FBS, GIBCO), L-glutamine (2 mM), penicillin (100 unites/mL), and streptomycin (100 µg/mL). All cells were incubated in a humidified atmosphere with 95% air and 5% CO₂ at 310 K.

E. Cellular-uptake experiment. HeLa cells (1.6 × 10⁵/ well) were cultured in 6 well plates overnight. After treated with different complexes (24 µM) for 3 and 8 h, cells were washed with ice-cold PBS 4 times, followed by incubated with milli-Q water (500 µL) in ice for 15 min. Cells were then harvested, 300 µL of which were
digested with HNO₃ (300 µL) overnight at room temperature, while the remaining 200 µL samples were kept for the protein assay. The digested samples were further diluted with milli-Q water to the appropriate concentration, and the gold analysis was performed on an Agilent 7500 inductively-coupled-plasma mass spectrometer (ICP-MS).

**F. Transmission electron microscopy.** After HeLa cells cultured in 10 cm cell culture dishes were treated with complex 1 (24 µM) for 24 h, MEM was discarded and cell mono-layer was washed with PBS (4 mL) once, followed by fixing with 2 mL ice cold glutaraldehyde (2.5% in 0.1 M sodium cacodylate-HCl buffer, pH 7.4) at 277 K overnight. Then, the cell mono-layer was washed by cacodylate buffer with 0.1 M sucrose, and was harvested to be fixed in osmium tetroxide (OsO₄) (1% in cacodylate buffer) for 30 min at room temperature, after which cells were embedded into agar, and further dehydrated with gradual increasing concentration of ethanol. Finally, cell pellets were embedded in epoxy resin polymerized at 333 K overnight, and were cut into ultrathin sections stained with uranyl acetate and lead citrate. The images of the sections were observed under a 208S Philips transmission electron microscopy.

**G. Cell transfection for vacuoles formation observation.** HeLa cells (2 × 10⁵ per 2 mL) were cultured in antibiotics free MEM supplemented with 10% FBS in 35 mm glass-bottomed microwell dishes (MatTek) and incubated at 310 K with 95% air and 5% CO₂. After 24 h, plasmid DNA of RFP (0.4 µg/mL) and YFP-ER (0.4 µg/mL,Yrbio) were co-transfected into cells following Lipofectamine 2000 protocol. When incubated for another 24 h, medium containing plasmid was replaced with fresh MEM with or without complex 1 (24 µM). After treated for 24 h, cell monolayer was washed with MEM gently and observed under a Zeiss
Axiovert 200M inverted fluorescence microscopy, and photos were taken by a Zeiss AxioCam MRc5 CCD camera.

**H. RNA extraction.** 8 × 10⁵ HeLa cells were cultured in 10 mL MEM in 10 cm dish overnight before treated with complex 1 (24 µM) for another 24 h. Then, total RNA was extracted using the RNeasy mini kit (Qiagen, CA) according to the instruction of the manufacturer, and was finally diluted with RNase free water (10 ng/µL).

**I. Oligonucleotide microarray analysis.** HeLa cells (8 × 10⁵) were plated in 10 cm dishes overnight, followed by treating with or without complex 1 (24 µM) in 10 mL MEN for 12 h and 24 h. The total RNA of the treated cells was extracted and purified using RNeasy kit (Qiagen). The cDNA microarray analysis was performed on an Affymetrix Human Genome U133 Plus 2.0 GeneChip. The data was analyzed with the Agilent GeneSpring GX software (Genome Research Centre, The University of Hong Kong).

**J. Reverse transcriptase-polymerase chain reaction (RT-PCR).** After mRNA was extracted, RT-PCR was performed by incubating mRNA (30 ng for CHOP, 20 ng for GRP78/Bip, and 10 ng for GAPDH), 0.1 µM of forward and reverse primers respectively and 1 µl of Superscript III RT/Platinum Taq Mix (Invitrogen) in RT-PCR mixture with a final volume of 25 µl at 331 K for 30 min, 367 K for 5 min, and followed by performing for 30 cycles (29 cycles for GRP78/Bip) consisting of 367 K for 1 min, 331 K for 1 min, and 341 K for 1 min. After the last cycle, the samples were heated for further 6 min at 341 K. The PCR products were separated with 1% agarose gel, and GAPDH was used as a probe for equal RCR products loading. All of the primers were purchased from Tech Dragon Ltd.
K. Western blotting analysis. After HeLa cells cultured in 10 cm dishes were treated with various chemicals for appropriate time, they were harvested and washed with ice-cold PBS twice before lysis buffer (20 mM Tris-HCl pH 7.2, 150 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Triton X-100, protease inhibitors) in ice for 15 min. Then, the lysate were centrifuged at 13,200 rpm for 5 min followed by denatured at 373 K for 10 min. The protein content for each sample was quantified by Protein Assay (Bio-Rad) and was normalized with lysis buffer. Proteins were fractionated on a 12% SDS-PAGE in a Tris-Glycine running buffer, and transferred onto a polyvinylidene fluoride (PVDF) membrane (GE Healthcare), which was later blocked in a TBST buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Tween 20) containing 5% bovine serum albumin (BSA, USB) at 277 K overnight. Then, the membrane was incubated with the primary antibodies at room temperature for 2 h, washed with TBST 3 times, and further incubated with secondary antibodies at room temperature for another 2 h. After washed with TBST, the immunoreactivity was examined with the enhanced chemiluminescence plus kit (GE Healthcare). GAPDH was used as a probe for the equal protein loading. GRP78/ Bip antibody was purchased from Santa Cruz Biotechnology, CHOP, Phospho-eIF2α, Phospho-PERK and HSP70 antibodies were purchased from Cell Signaling.

L. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. HeLa cells (1.6 × 10^5/ well) were cultured in 6 well plates overnight followed by treated with complex 1 for 24 h. After washed with PBS, cells were treated with the APO-BrdUTM TUNEL Assay Kit (Invitrogen) according to the manufacturer’s instruction. HeLa cells that were treated with DNase I (300 U/ mL in 50 mM Tris-HCl pH 7.5, 1 mg/ mL BSA in PBS) which induce DNA stand breaking at room temperature for 10 min were used as positive controls.
**M. Hoechst 33342 nuclear staining experiment.** HeLa cells \((1.5 \times 10^5)\) in 2 mL were cultured in 35 mm glass-bottomed microwell dishes (MatTek) overnight before treated with complex 1 \((24 \mu M, 24 \text{ h})\). After treatment, cells were stained with Hoechst 33342 \((5 \mu g/ \mu l)\) at 310 K for 5 min. The stained cells were observed with a Zeiss Axiovert 200M inverted fluorescence microscopy.

**N. Cell-cycle analysis.** HeLa cells were plated in 10 cm dishes with a density of \(8 \times 10^5 \text{ cells/ 10 mL}\). After incubated 310 K in a humidified atmosphere of 5% CO\(_2\)/95% air for 24 h, MEM was replaced with 10 mL fresh MEM with or without complex 1 \((24 \mu M)\). After incubated or another 24 h, cells were harvested, trypsinized and washed with pre-cold PBS \((1\times, 5 \text{ mL})\) once, followed by fixing with ice-cold ethanol \((70\%, 2 \text{ mL})\) for 1 h at 277 K. Cells were further washed with PBS containing 1% BSA twice, incubated with RNase A \((30 \mu g/ \mu l)\) in PBS at 310 K for 1 h, and stained in propidium iodide \((40 \mu g/ \mu l)\) in PBS at room temperature for 30 min in dark. \(3 \times 10^4\) cells were counted in a Coulter EPICS flow cytometer (Coulter, Miami, FL) equipped with 480 long, 525 band, and 625 long pass mirrors. The data was analyzed with Modfit 5.11 software.

**O. Tube-formation assay.** The tube-formation assay was conducted following the *In Vitro* Angiogenesis Kit (Millipore Corporation). \(10\times\) Diluent Buffer was mixed with ECMatrixTM solution in the ratio of 1:9, and 50 \(\mu\)L of the mixture was transferred to each well of 96 well plates which was further incubated at 310 K for 1 h allowing polymerization. Then, MS1 cells \((80,000)\) mixed with different concentration of complex 1 \((0, 6, 12, 24 \mu M)\) in 100 \(\mu\)L DMEM medium containing Endothelial Cell Growth Supplements (ECGS) \((0.05 \mu g/ \mu l)\) was added on the top of the polymerized matrix. After incubated at 310 K for 2 h, tube formation was investigated with a Zeiss Axiovert 200M inverted fluorescence microscopy at a \(5\times\) magnification.
2. Figures and tables

Fig. S1. The 2-D H-H (COSY) spectrum of 1 in DMSO-$d_6$. 
Fig. S2. The positive-ion FAB mass spectrum of 1.
Fig. S3. The 2-D H-H (COSY) spectrum of 2 in DMSO-$d_6$. 
Fig. S4. The positive-ion FAB mass spectrum of 2.
Fig. S5. The 2-D H-H (COSY) spectrum of 3 in DMSO-\textit{d}_6.
Fig. S6. The positive-ion FAB mass spectrum of 3.
Fig. S7. The 2-D H-H (COSY) spectrum of 4 in DMSO-$d_6$. 
Fig. S8. The positive-ion FAB mass spectrum of 4.
Fig. S9. The UV-vis spectral traces of 1–4 in PBS (pH 7.4) for 72 h.
Fig. S10. The ESI-MS study revealed the peaks of \([(\text{BCN})\text{Au}^{\text{III}}(\text{GSH})_2-2\text{H}]^{2-}\) and \([(\text{BCN})\text{Au}^{\text{III}}(\text{GSH})_2-\text{H}]^-\) in the solution (A) and (B) \([(\text{BCN})\text{Au}^{\text{III}}(\text{GSH})-\text{H}]^-\) in the precipitate after \(1\) was incubated with GSH (pH 7.4, 2 mM).
**Fig. S11.** Cellular uptake (µg/ µg protein) of gold as quantified by ICP-MS experiments for HeLa cells treated with 1 (24 µM) and 2 (24 µM) for 3 and 8 h.
Fig. S12. The cytotoxic profiles of 1 towards HeLa and normal lung fibroblast (CCD-19Lu) cell lines.
**Fig. S13.** Cytoplasmic vacuolization (examples are indicated by white arrows) induced by 1 (24 µM, 24 h) in HeLa cells observed with light microscopy.
**Fig. S14.** Fold changes (vs vehicle control) of ER-stress related mRNAs expression in HeLa cells treated with 1 (24 µM) for 12 and 24 h in the oligonucleotide microarray analysis.
Fig. S15. The inhibition effect of the caspase inhibitor z-VAD-fmk on the cytoplasmic vacuolization (A) and cell death (B) induced by 1 (24 µM, 24 h) in HeLa cells.
**Fig. S16.** Apoptotic study using TUNEL assay. (A)–(C) Vehicle-treated cells were used as negative controls. (D)–(F) Complex 1 (24 µM, 24 h)-treated HeLa cells. (G)–(I) DNase I pretreated cells as positive control. Left column: TUNEL images; middle column: propidium iodide (PI) images; right column: merged images of TUNEL and PI.
**Fig. S17.** Fluorescence images of Hoechst 33342-stained HeLa cells in the (A) absence, or (B) presence of 1 (24 μM, 24 h), or (C) presence of cisplatin (20 μM, 24 h).
Fig. S18. The protein expression levels of cleaved caspase-3, cleaved caspase-7 and cleaved PARP for HeLa cells treated with 1 (24 µM, 24 h) was evaluated by western blotting experiment. HeLa cells treated with cisplatin (20 µM) for 24 h were chosen as positive control.
**Fig. S19.** S-phase cell-cycle arrest induced by 1 (24 µM) in HeLa cells for 24 h examined by flow cytometric analysis.
Fig. S20. (A) Cytoplasmic vacuolization (examples are indicated with white arrows) was inhibited by cycloheximide (CHX, 10 µM). (B) Inhibition of HeLa cell death by CHX (10 µM) in the presence of 1 for 24 h was observed by means of MTT assay.
Fig. S21. (A) Tube-formation assay showing the anti-angiogenic effect of 1 towards MS1 cells with incubation time of 2 h. (B) Parallel cytotoxicity evaluation of 1 toward MS1 cells for 2 h by means of MTT assay.
Table 1. The IC\textsubscript{50} values (µM, 72 h) of 1–4 toward various cell lines by MTT assay.

<table>
<thead>
<tr>
<th></th>
<th>HeLa\textsuperscript{a}</th>
<th>B16\textsuperscript{b}</th>
<th>PLC\textsuperscript{c}</th>
<th>MDA-MB-231\textsuperscript{d}</th>
<th>SUNE1\textsuperscript{e}</th>
<th>U87\textsuperscript{f}</th>
<th>CCD-19Lu\textsuperscript{g}</th>
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<tbody>
<tr>
<td><strong>1</strong></td>
<td>7.7±0.1</td>
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<td>8.8±0.3</td>
<td>8.7±0.2</td>
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<td>17.1±0.3</td>
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<tr>
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<td>12.0±2.6</td>
<td>35.6±1.1</td>
<td>36.2±0.4</td>
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\textsuperscript{a}Cervical epithelial carcinoma. \textsuperscript{b}Melanoma. \textsuperscript{c}Hepatocellular carcinoma. \textsuperscript{d}Breast carcinoma. \textsuperscript{e}Nasopharyngeal carcinoma. \textsuperscript{f}Glioblastoma. \textsuperscript{g}Normal lung fibroblasts.
3. References
