Electronic Supplementary Information (ESI)

For

Anticancer gold(I)-phosphine complexes as potent autophagy-inducing agents

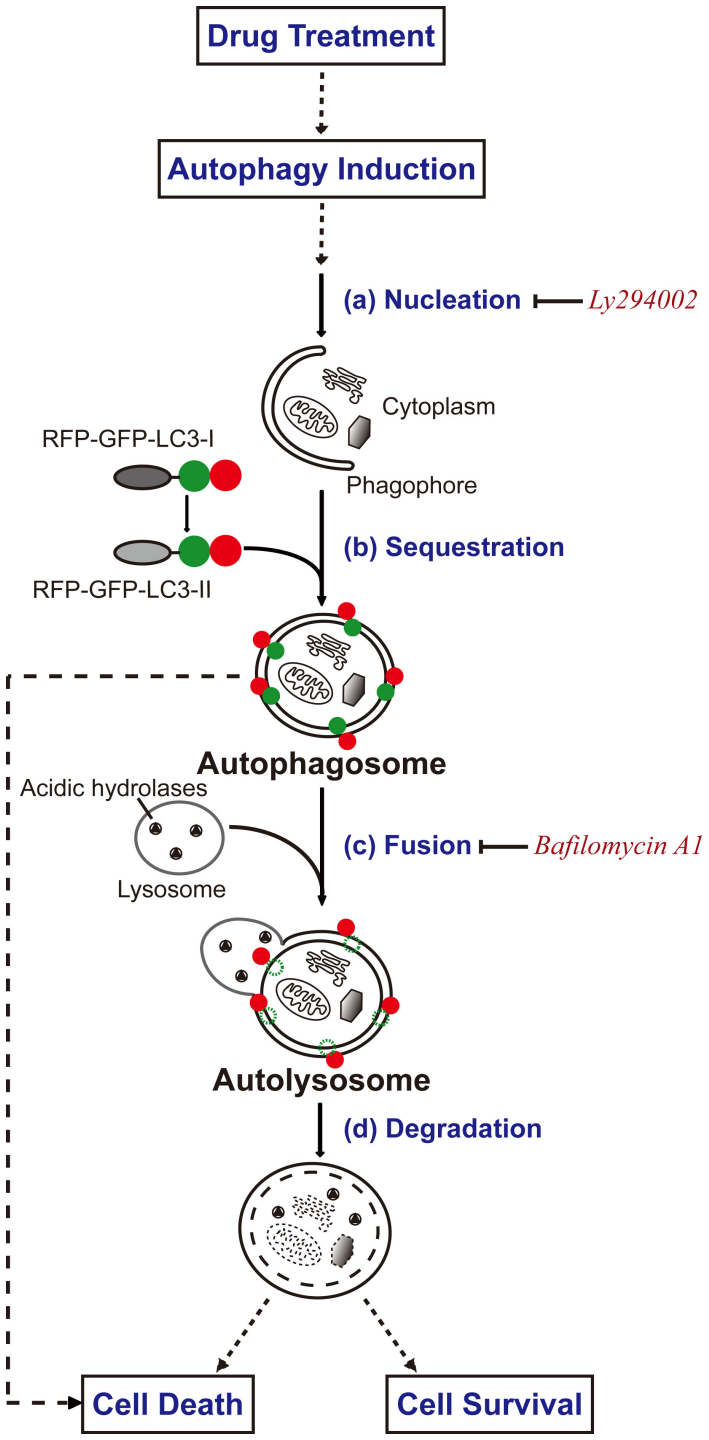
Songhai Tian, Fung-Ming Siu, Steven C. F. Kui, Chun-Nam Lok

and Chi-Ming Che*

Department of Chemistry, State Key Laboratory on Synthetic Chemistry and Open Laboratory of Chemical Biology of the Institute of Molecular Technology for Drug Discovery and Synthesis, The University of Hong Kong, Pokfulam Road, Hong Kong, China. E-mail: cmche@hku.hk; Fax: (+852) 2857-1586; Tel: (+852) 2859-2154

Table of contents

1. Schematic diagram of autophagic pathway.



(Modified from ref. 4)

Fig. S1. Schematic diagram of autophagic pathway. Macroautophagy (referred to as autophagy) is an intracellular catabolic process in which cytoplasmic components are sequestrated within autophagosomes for bulk degradation by lysosomes.[1, 2] In the circumstances of nutrient deprivation or drugs treatment, autophagy could be induced

both *in vitro* and in *vivo*.[3] There are four major steps in the autophagic pathway after autophagy induction:

(a) Vesicle nucleation. Vesicle nucleation is characterized by the formation of cup-shaped, membrane-bounded phagophores.[4] This process requires type III phosphatidylinositol 3-kinase (PI3K) and involves a large number of evolutionarily conserved autophagy-related proteins (ATGs). *Ly294002* (Ly2), an PI3K inhibitor,[5] is used to inhibit this step in our studies. Ly2-pretreatment prevents phagophores formation and reduces the total amounts of autophagosomes and autolysosomes.

(b) Autophagosomes sequestration. Upon phagophores expansion,[6, 7] bulk cytoplasm (including dysfunctional mitochondria, fractured endoplasmic reticulum, mis-folded proteins and so on) is enclosed and sequestrated forming autophagosomes. Under the transmission electron microscopy, autophagosomes are visualized as double-membrane bounded vacuoles, containing degenerating components (Fig. 2 in main text).[8] Autophagosomes formation can also be examined by the translocation of microtubule-associated protein 1 Light Chain 3 (LC3) from cytoplasm to the membrane of autophagosomes.[7, 9] HeLa cells stably transfected with a marker gene, tandem fluorescent-tagged LC3 (RFP-GFP-LC3, tfLC3)[10] was employed to monitor the LC3 translocation. In the absence of autophagy induction, the LC3 fluorescent signals are evenly distributed; upon autophagy induction, the punctate fluorescent signals (LC3 dots) appear as a result of LC3 accumulated on the membrane of autophagosomes (Fig. 1A in main text). The formation of autophagosomes can also be examined by immunoblotting analysis of the post-translational processing of LC3 proteins (Fig. 1C in main text). The level of processed form of LC3 (LC3-II) serves as a read-out of the amount of autophagosomes.

(c) Autolysosomes formation. The autophagosomes eventually fuse with lysosomes to form autolysosomes. As the GFP signals are quenched at acidic pH whereas the RFP signals are insensitive to pH changes, the GFP signals disappear within the acidic lysosomal environment which the RFP signals persist. As a result, the red fluorescent dots (RFP-LC3 dots) indicate the total of autophagosomes and autolysosomes, and the co-localized green (GFP) and red (RFP) fluorescent dots (display as yellow dots in overlapped GFP and RFP images) represent the autophagosomes. In the present study, the percentage of cells with significant RFP-LC3 dots was used to represent the LC3 translocation and to measure the autophagy level. *Bafilomycin A1* (Baf), an inhibitor of vacuolar H^+-ATPase[11], is used to inhibit the fusion step in our studies. The pretreatment of Baf prevents the formation of autolysosomes and leads to accumulation of autophagosomes.[11]

(d) Autolysosomes degradation. Both the inner membrane of autophagosome and its luminal content will be degraded by lysosomal acidic hydrolases. The finishing of degradation marks the completion of the autophagic pathway.

However, autophagy may have two divergent consequences for cancer cells: cell survival or cell death.[12, 13] In recent reports, the accumulation of autophagosomes is associated with cell death, implying that the autolysosomes degradation step may be unnecessary.[14, 15]

2. Experimental

a. Materials

i. Chemicals and reagents. dppm (bis (biphenylphosphino)methane) and dpmp (bis (diphenylphosphinomethyl)phenylphosphine) were purchased from the Strem Chemicals Inc. (Newburyport, MA, USA). All the other chemicals that were used in synthesis were purchased from Sigma-Aldrich Chemical Co. (St Louis, MI, USA), unless otherwise stated. Cisplatin (*cis*-diammineplatinum(II) dichloride, Sigma, P4394), MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, Sigma, M2128), Rapamycin (Sigma, R0395), Ly294002 (Sigma, L9908), Bafilomycin A1 (Sigma, B1793) were used. Antibodies, rabbit anti-LC3 (Abgent, AP1802a), rabbit anti-GAPDH (Cell Signaling, #2118), sheep anti-rabbit IgG HRP-linked antibody (GE Healthcare, NA931V) were used. **Au-1** ($[Au(TU)_2]Cl$) was prepared as stated in our previous study.[16] **Au-2** ([Au(1,3-bis(2,6-di-*i*-propylphenyl)imidazol-2-ylidene)]Cl) was prepared according to literature procedures.[17]

ii. Synthesis of Au-3. **Au-3** ($[(C\text{^}N\text{^}C)Au_3(\mu\text{-dppm})_2]Cl$) was prepared as stated in our previous study.[18] ^{1}H NMR (400 MHz, CD_2Cl_2): δ 1.22 (m, 2H, H^a), 4.36 (m, 2H, H^b), 6.54 (d, 2H, H^6), 6.86 (t, 2H, H^3), 6.91–7.00 (m, 10H, H^5, H^{16} and H^{24}), 7.06–7.10 (m, 6H, H^4, H^{17} and H^{25}), 7.18–7.3 (m, 8H, H^{19} and H^{20}), 7.33 (d, 2H, H^8), 7.25–7.51 (m, 6H, H^{21} and H^{23}), 7.57 (dd, 4H, H^{15}), 7.69–7.79 (m, 7H, H^9, H^{12} and H^{13}), 8.00 (dd, 4H, H^{11}). ^{31}P NMR (162 MHz, CD_2Cl_2): δ 30.98 (dd), 37.26 (dd). MS (FAB, +ve): 1589 (M^+). Anal. Calcd for $C_{67}H_{55}NP_4Au_3Cl \cdot 1/2CHCl_3$: C, 48.10; H, 3.32; N, 0.838. Found: C, 48.59; H, 3.35; N, 1.01.

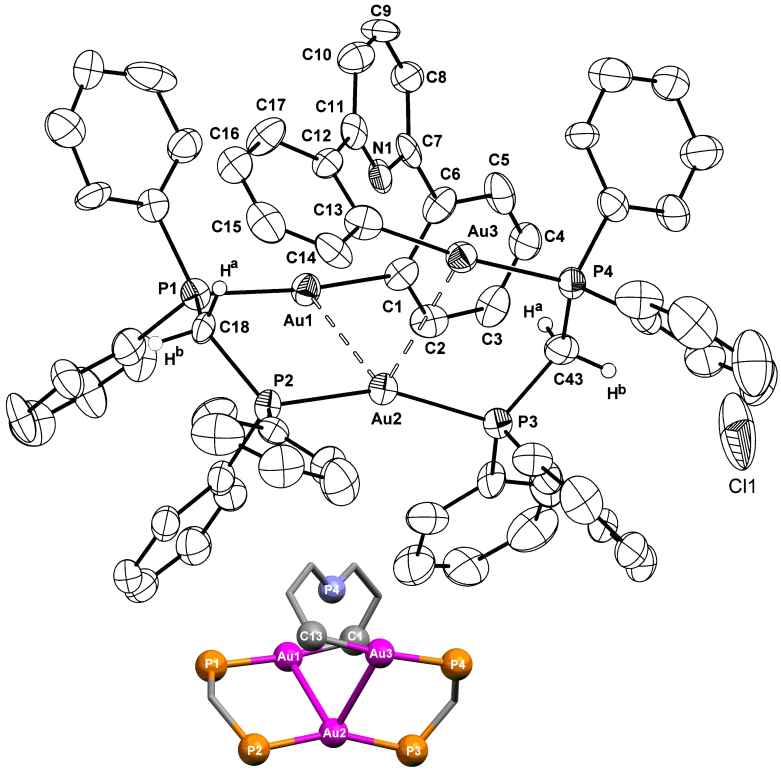


Fig. S2. Perspective view of **Au-3** ([(C^N^C)Au$_3$(μ-dppm)$_2$]Cl). For clarity, all the hydrogen atoms are omitted (except four methylene hydrogen atoms). Non-hydrogen atoms are represented anisotropically by thermal ellipsoids drawn at 30 % probability level.

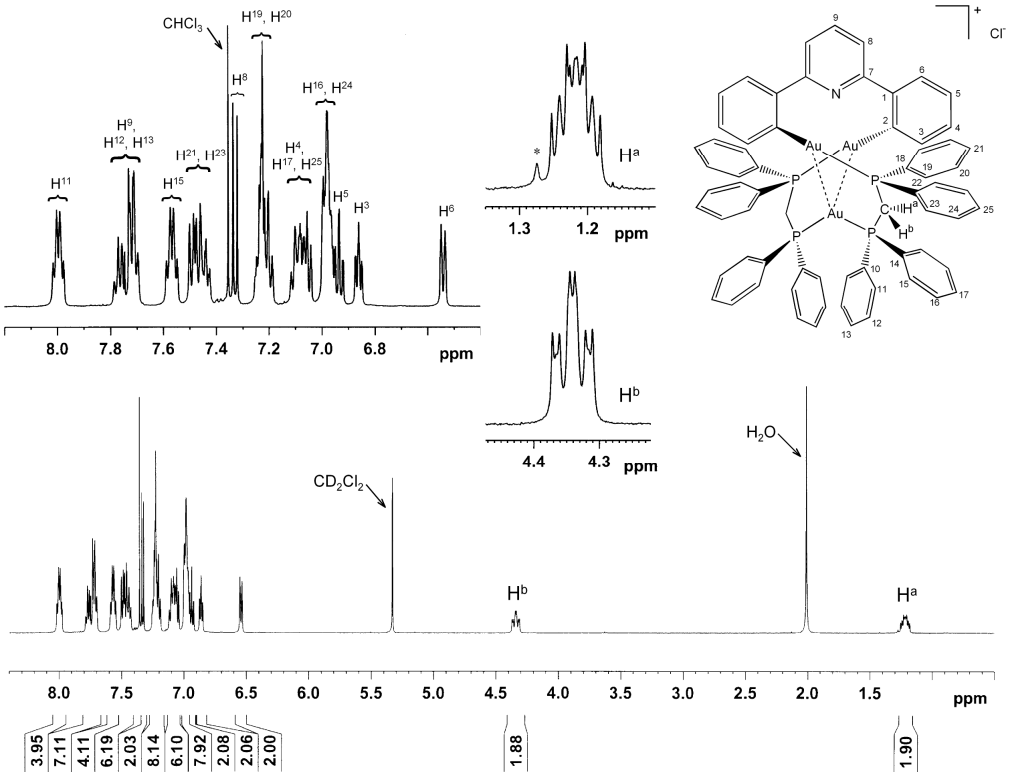


Fig. S3. ^{1}H NMR spectrum for **Au-3** showing proton H^a shifted to up-field (1.22 ppm) when compared with the non-shielded proton H^b (4.36 ppm) in CD_2Cl_2.

iii. Synthesis of Au-4a. Au-4a ([Au(PPh_3)Cl]) was prepared according to literature procedures.[19] ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.48–7.54 (m, 15H). ^{31}P NMR (162 MHz, $CDCl_3$): δ 33.30 (s). MS (FAB, +ve): 494 (M^+). Anal. Calcd for $C_{18}H_{15}AuClP$: C, 43.70; H, 3.06; Found: C, 43.75; H, 3.15.

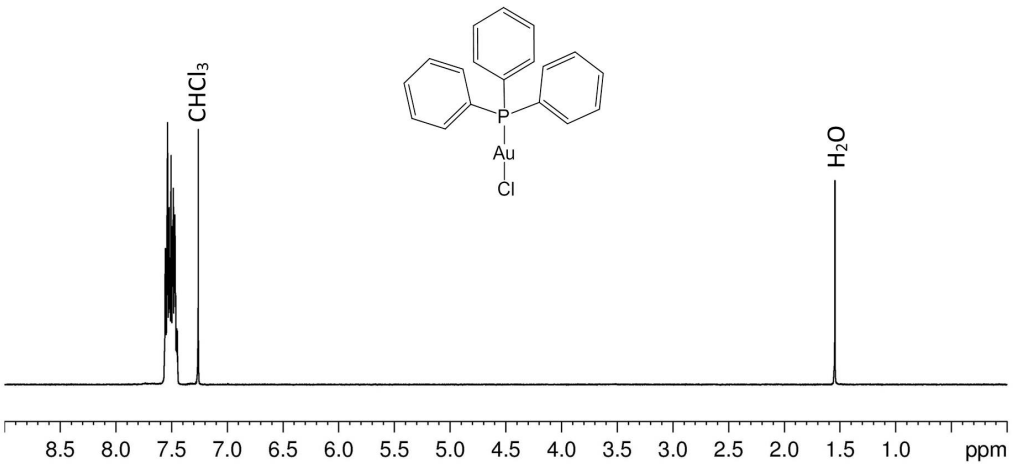


Fig. S4. ^{1}H NMR spectrum of **Au-4a** in $CDCl_3$.

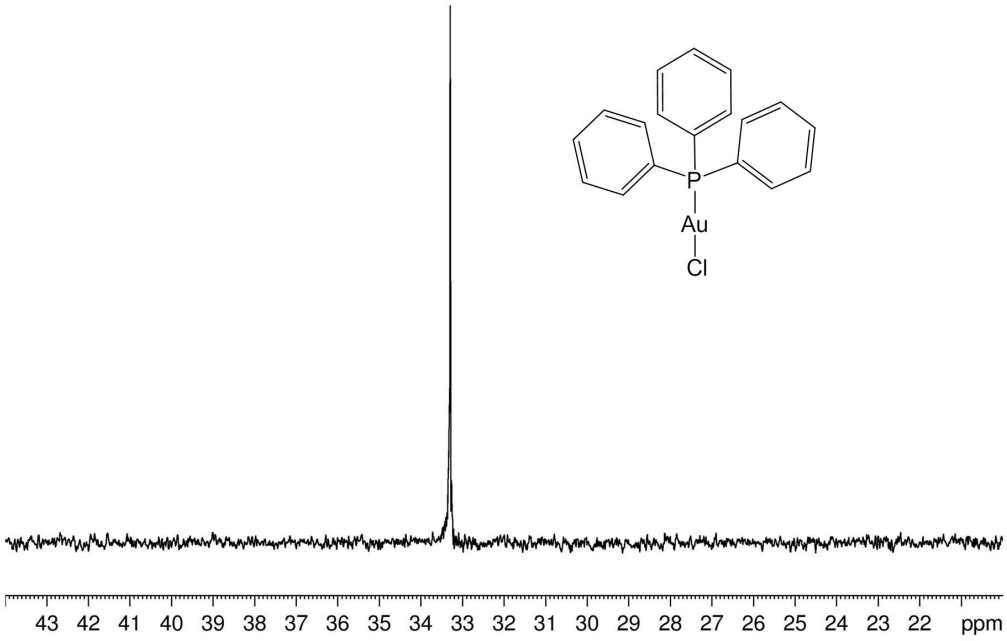


Fig. S5. ^{31}P NMR spectrum of **Au-4a** in $CDCl_3$.

iv. Synthesis of Au-4b. Au-4b ([Au_2(μ-dppm)Cl_2]) was prepared according to literature procedures.[20] ^{1}H NMR (400 MHz, d_6-DMSO): δ 4.67 (t, 2H), 7.43 (t, 8H, H^2), 7.51 (t, 4H, H^3), 7.76 (m, 8H, H^1). ^{31}P NMR (162 MHz, d_6-DMSO): δ 27.27 (s). MS (FAB, +ve): 848 (M^+). Anal. Calcd for $C_{25}H_{22}Au_2Cl_2P_2$: C, 35.36; H, 2.61; Found: C, 35.42; H, 2.66.

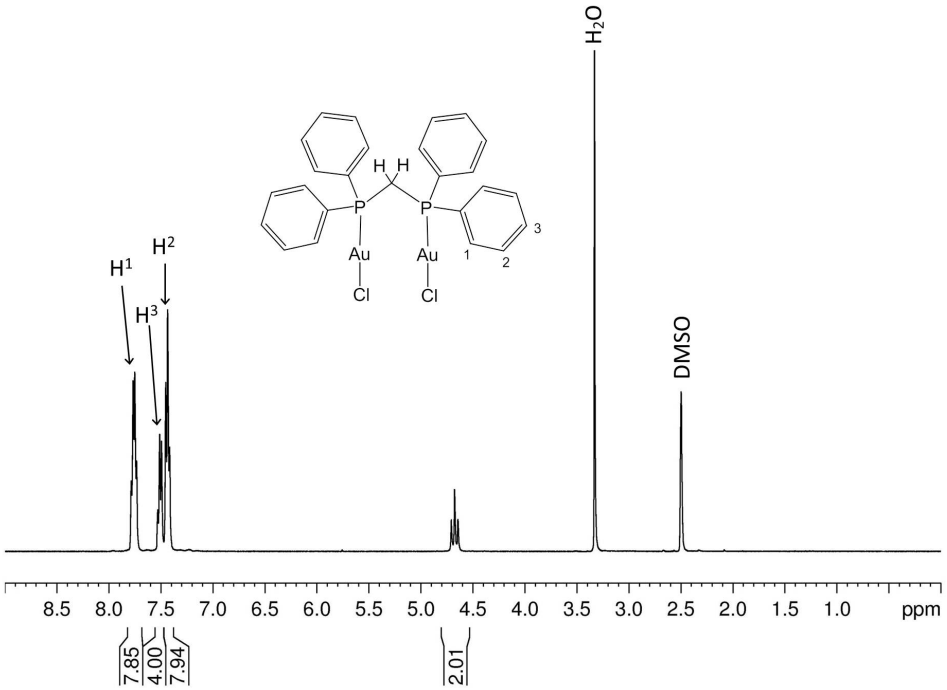


Fig. S6. ^{1}H NMR spectrum of **Au-4b** in d_6-DMSO.

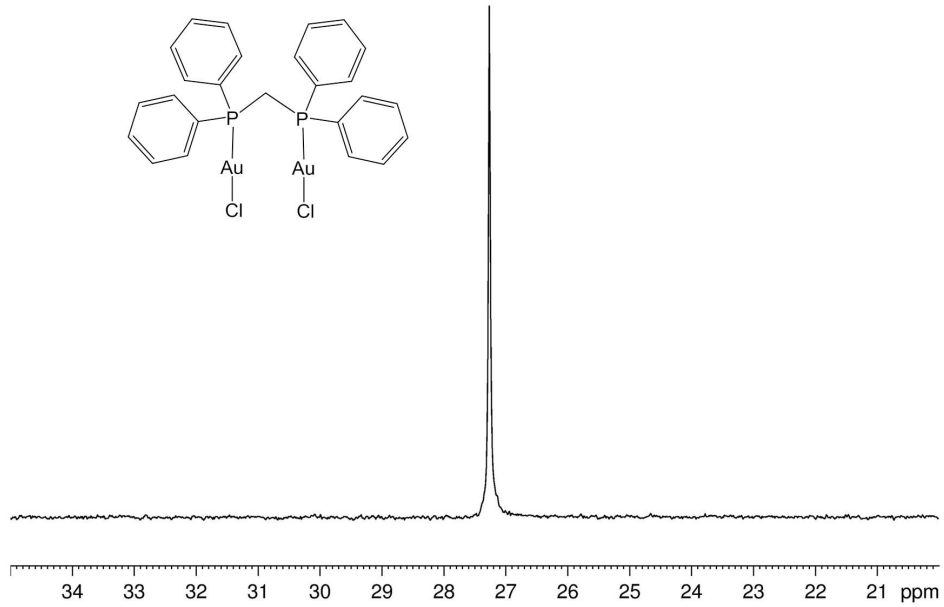


Fig. S7. ^{31}P NMR spectrum of **Au-4b** in d_6-DMSO.

v. Synthesis of Au-4c. Au-4c ([Au_3(μ-dpmp)Cl_3]) was prepared according to literature procedures.[21] ^{1}H NMR (400 MHz, d_6-DMSO): δ 4.10–4.19 (m, 2H), 4.24–4.34 (m, 2H), 7.18–7.23 (t, 2H), 7.32–7.36 (t, 4H), 7.42–7.47 (m, 4H), 7.55–7.66 (m, 11H), 7.73–7.78 (m, 4H). ^{31}P NMR (162 MHz, d_6-DMSO): δ 21.86 (t), 24.90 (d). MS (FAB, +ve): 1202 (M^+). Anal. Calcd for $C_{32}H_{29}Au_3Cl_3P_3$: C, 31.93; H, 2.43; Found: C, 32.03; H, 2.46.

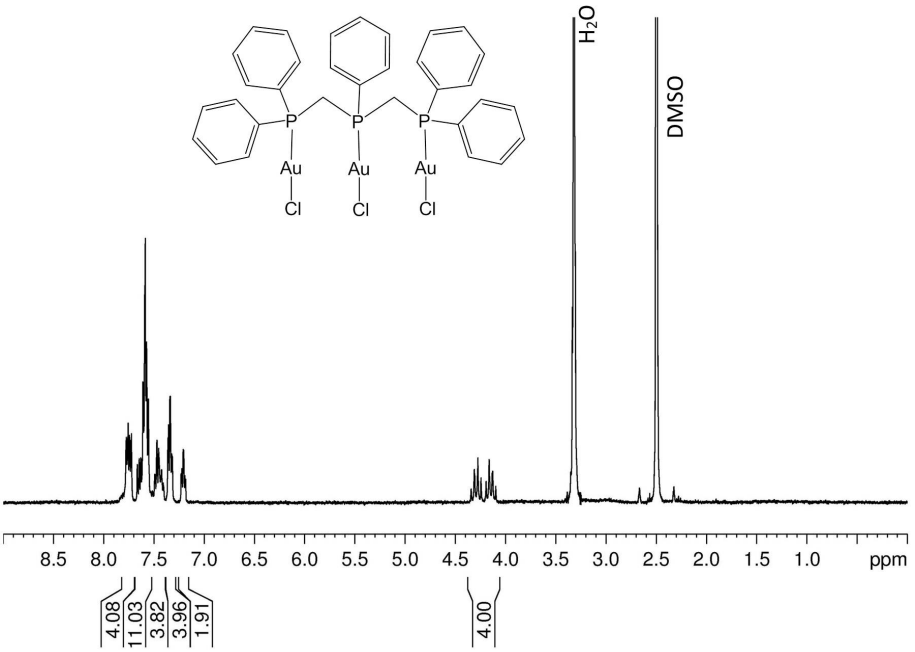


Fig. S8. ^{1}H NMR spectrum of **Au-4c** in d_6-DMSO.

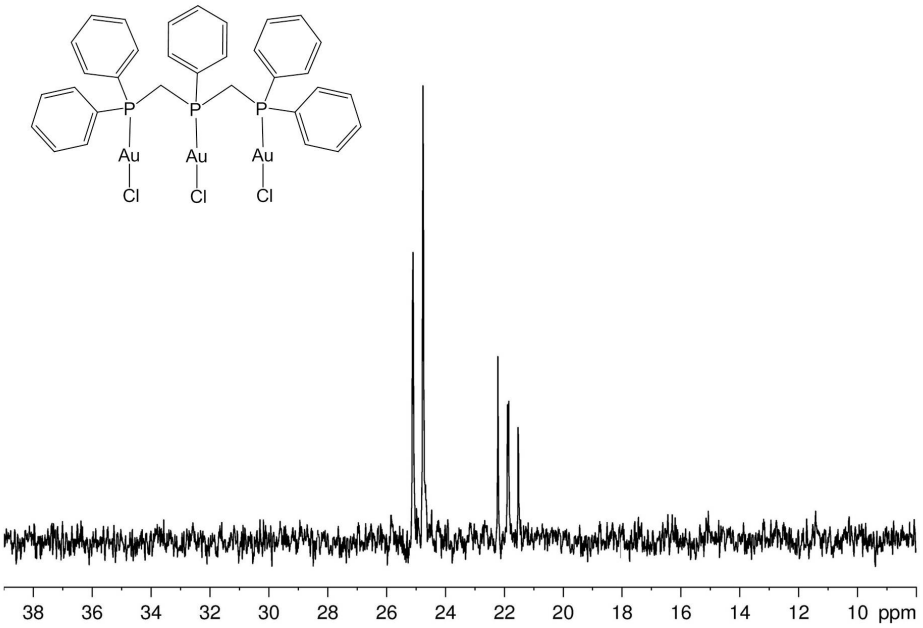


Fig. S9. ^{31}P NMR spectrum of **Au-4c** in d_6-DMSO.

b. Cell culture

HeLa, NCI-H460, MDA-MB-231, SUNE1, HepG2, SW480, PLC and HL-60 cells were obtained from American Type Culture Collection. The tfLC3 cells were the HeLa cells stably transfected with plasmid encoding RFP-GFP-LC3[10] (gift from Prof. Tamotsu Yoshimori, Department of Genetics, Osaka University Graduate School of Medicine, Osaka, Japan). HeLa and tfLC3 cells were maintained in Minimum Essential Medium (MEM, GIBCO) supplemented with 10% Fetal Bovine Serum (FBS, GIBCO), L-glutamine (2 mmol/L), penicillin (100 unites/mL), and streptomycin (100 μg/mL). NCI-H460, MDA-MB-231, SUNE1, HepG2, SW480, PLC and HL-60 cells were maintained in RPMI-1640 Medium (Sigma, R4130-1L) supplemented with 10% Fetal Bovine Serum (FBS, GIBCO) and L-glutamine (2 mmol/L). Cultures were incubated in a humidified atmosphere of 95 % air and 5 % CO_2 at 37 °C.

c. Cell viability assay

The gold(I) complexes, dppm and cisplatin were dissolved in DMSO as a 10 mM stock before use. HeLa cells (about 8×10^3/well), NCI-H460 cells (about 1.5×10^4/well), MDA-MB-231 cells (about 4×10^4/well) , SUNE1 (about 1.5×10^4/well), HepG2 (about 3×10^4/well), SW480 (about 4×10^4/well), PLC (about 8×10^4/well) and HL-60 cells (about 8×10^4/well) were seeded in 96-well flat-bottomed microplates in supplemented medium and incubated for 24 h. The medium was replaced with 100 μL drug-containing medium (serial dilution of each complex from 100 μM to 0.2 μM), and cells were further incubated for 48 h. Then 10 μL MTT (5 mg/mL in water) was added to each well and incubated for 4 h, 100 μL solubilization solution (10% SDS in 0.01 M HCl) was added to each well for 24 h. The absorbance of formazan formed was measured at 580 nm by a microplate analyzer (Perkin-Elmer Fusion α-FP). A vehicle-control (0.2% DMSO) was run in parallel.

d. Autophagy assays[22]

i. Autophagy-treatment conditions. tfLC3 cells (2×10^5) were plated in 35-mm glass-bottomed microwell dishes (MatTek) with supplemented medium 24 h before gold(I) complexes treatment. According to the cytotoxicities, early stage of treatment (1 μM at 24 h) which is before the cell death was chosen as the experimental conditions for the assays. 250 nM Rapamycin (Rapa, 1 mM stock solution in DMSO) was used as positive control of autophagy. For the experiments using autophagy blockers, tfLC3 or HeLa cells were pretreated with 10 μM Ly294002 (Ly2, 10 mM stock solution in DMSO) or 15 nM Bafilomycin A1 (Baf, 100 μM stock solution in DMSO) for 2 h, and then treated with **Au-4b** or Rapa. The vehicle control (DMSO) was studied in parallel for comparison.

ii. Fluorescence microscopy analysis. The tfLC3 cells were examined using the Zeiss Axiovert 200M inverted fluorescence microscopy. The images are captured by a Zeiss AxioCam MRc5 CCD camera with Zeiss AxioVision Rel. 4.5 software. Fluorescent images are analyzed by the software ImageJ. "Cells with RFP-LC3 dots" in the main text and figure legends is defined as cells with at least 50 punctate red fluorescent dots, which indicates the accumulation of autophagosomes and autolysosomes. "Cells with RFP-GFP-LC3 dots" is defined as cells with at least 20 co-localized red and green fluorescent dots, which indicates the accumulation of autophagosomes. The percentages of cells with RFP-LC3 or RFP-GFP-LC3 dots were counted in 6 random non-overlapping fields. Experiments were repeated at least three times for the calculation of standard deviation.

iii. Western blotting.[23] HeLa cells were treated with lysis buffer (20 mM Tris, pH 7.2, 150 mM NaCl, 2 mM EDTA, 10 % glycerol, 1 % Triton X-100, protease inhibitors). The lysate was centrifuged at 10,000 × g for 10 min followed by heat denaturation for 5 min. Proteins were separated by 12 % SDS-PAGE, and transferred onto a polyvinylidene fluoride (PVDF) membrane (GE Healthcare). The membrane was blocked with a TBST buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 0.1 % Tween-20) containing 5 % bovine serum albumin (BSA, USB) at 4 ℃ overnight. Then the membrane was incubated with the primary antibodies at room-temperature for 2 h, washed and incubated with secondary antibodies for 2 h. The immunoreactivities were detected with the enhanced chemiluminescence plus kit (GE Healthcare). GAPDH was probed to ensure equal protein loading. Quantitation analysis was done with the software ImageJ.

iv. Transmission electron microscopy. HeLa cells treated with various gold(I) complexes were harvested and washed with PBS. The cells were fixed by ice-cold glutaraldehyde (2.5% in 0.1 M cacodylate buffer, pH 7.4) at 4 □ overnight. After washing with cacodylate buffer, cells were post-fixed in 1 % OsO_4 (w/v, pH 7.2) for 30 min at room-temperature. After gradual dehydration with increasing concentration of ethanol, cells were embedded into agar and polybed resin. Ultrathin sections were doubly stained with uranyl acetate and lead citrate. The images were examined with a 208S Philips transmission electron microscopy.

e. Statistical analysis

Each experiment was repeated at least three times for the standard deviation calculation. The statistical significance of the differences was assessed using paired t-test of Office Excel 2003 (Microsoft) with p value less than 0.05 to be considered statistically significant.

3. References

1 A. L. Edinger and C. B. Thompson, *Cancer Cell*, 2003, **4**, 422-424.

2 D. C. Rubinsztein, J. E. Gestwicki, L. O. Murphy and D. J. Klionsky, *Nat. Rev. Drug. Discov.*, 2007, **6**, 304-312.

3 B. Levine and G. Kroemer, *Cell*, 2008, **132**, 27-42.

4 Z. Xie and D. J. Klionsky, *Nat. Cell. Biol.*, 2007, **9**, 1102-1109.

5 E. F. C. Blommaart, U. Krause, J. P. M. Schellens, H. Vreeling-Sindelárová and A. J. Meijer, *Eur. J. Biochem.*, 1997, **243**, 240-246.

6 K. Suzuki, T. Kirisako, Y. Kamada, N. Mizushima, T. Noda and Y. Ohsumi, *EMBO J.*, 2001, **20**, 5971-5981.

7 A. Orsi, H. EJ. Polson and S. A. Tooze, *Curr. Opin. Cell. Biol.*, 2010, **22**, 150-156.

8 G. Kroemer, L. Galluzzi, P. Vandenabeele, J. Abrams, E. S. Alnemri, E. H. Baehrecke, M. V. Blagosklonny, W. S. EI-Deiry, P. Golstein, D. R. Green, M. Hengartner, R. A. Knight, S. Kumar, S. A. Lipton, W. Malorni, G. Nuñez, M. E. Peter, J. Tschopp, J. Yuan, M. Piacentini, B. Zhivotovsky and G. Melino, *Cell. Death. Differ.*, 2009, **16**, 3-11.

9 M. C. Maiuri, E. Zalckvar, A. Kimchi and G. Kroemer, *Nat. Rev. Mol. Cell. Bio.*, 2007, **8**, 741-752.

10 S. Kimura, T. Noda and T. Yoshimori, *Autophagy*, 2007, **3**, 452-460.

11 A. Yamamoto, Y. Tagawa, T. Yoshimori, Y. Moriyama, R. Masaki and Y. Tashiro, *Cell. Struct. Funct.*, 1998, **23**, 33-42.

12 M. C. Maiuri, L. Galluzzi, J. M. Vicencio, O. Kepp, E. Tasdemir and G. Kroemer, *Curr. Mol. Med.*, 2008, **8**, 78-91.

13 E. Tasdemir, L. Galluzzi, M. C. Maiuri, A. Criollo, I. Vitale, E. Hangen, N. Modjtahedi and G. Kroemer, *Methods Mol. Biol.*, 2008, **445**, 29-76.

14 L. Jahreiss, M. Renna, R. Bittman, G. Arthur and D. C. Rubinsztein, *Autophagy*, 2009, **5**, 835-846.

15 S. Tian, J. Lin, J. Jun Zhou, X. Wang, Y. Li, X. Ren, W. Yu, W. Zhong, J. Xiao, F. Sheng, Y. Chen, C. Jin, S. Li, Z. Zheng and B. Xia, *Autophagy*, 2010, **6**, 1032-1041.

16 K. Yan, C.-N. Lok, K. Bierla and C.-M. Che, *Chem. Commun.*, 2010, **46**, 7691-7693.

17 P. de Frémont, N. M. Scott, E. D. Stevens and S. P. Nolan, *Organometallics*, 2005, **24**, 2411-2418.

18 S. C. F. Kui, J.-S. Huang, R. W.-Y. Sun, N. Zhu and C.-M. Che, *Angew. Chem., Int. Ed.*, 2006, **45**, 4663-4666.

19 M. I. Bruce, B. K. Nicholson and O. B. Shawkataly, *Inorg. Synth.*, 1989, **26**, 324-328.

20 M. C. Brandys, M. C. Jennings and R. J. Puddephatt, *J. Chem. Soc.*, *Dalton Trans.*, 2000, 4601-4606.

21 M. Bardají, A. Laguna, V. M. Orera and M. D. Villacampa, *Inorg Chem.*, 1998, **37**, 5125-5130.

22 D. J. Klionsky, H. Abeliovich, P. Agostinis, D. K. Agrawal, G. Aliev, D. S. Askew, M. Baba, E. H. Baehrecke, B. A. Bahr, A. Ballabio, B. A. Bamber, D. C. Bassham, E. Bergamini, X. Bi, M. Biard-Piechaczyk, J. S. Blum, D. E. Bredesen, J. L. Brodsky, J. H. Brumell, U. T. Brunk, W. Bursch, N. Camougrand, E. Cebollero, F. Cecconi, Y. Chen, L. S. Chin, A. Choi, C. T. Chu, J. Chung, P. G. H. Clarke, R. S. B. Clark, S. G. Clarke, C.

Clave, J. L. Cleveland, P. Codogno, M. I. Colombo, A. Coto-Montes, J. M. Cregg, A. M. Cuervo, J. Debnath, F. Demarchi, P. B. Dennis, P. A. Dennis, V. Deretic, R. J. Devenish, F. Di Sano, J. F. Dice, M. Difiglia, S. Dinesh-Kumar, C. W. Distelhorst, M. Djavaheri-Mergny, F. C. Dorsey, W. Dröge, M. Dron, W. A. Dunn, Jr., M. Duszenko, N. T. Eissa, Z. Elazar, A. Esclatine, E. L. Eskelinen, L. Fésüs, K. D. Finley, J. M. Fuentes, J. Fueyo, K. Fujisaki, B. Galliot, F. B. Gao, D. A. Gewirtz, S. B. Gibson, A. Gohla, A. L. Goldberg, R. Gonzalez, C. González-Estévez, S. Gorski, R. A. Gottlieb, D. Häussinger, Y. W. He, K. Heidenreich, J. A. Hill, M. Høyer-Hansen, X. Hu, W. P. Huang, A. Iwasaki, M. Jäättelä, W. T. Jackson, X. Jiang, S. Jin, T. Johansen, J. U. Jung, M. Kadowaki, C. Kang, A. Kelekar, D. H. Kessel, J. A. K. W. Kiel, H. P. Kim, A. Kimchi, T. J. Kinsella, K. Kiselyov, K. Kitamoto, E. Knecht, M. Komatsu, E. Kominami, S. Kondo, A. L. Kovács, G. Kroemer, C. Y. Kuan, R. Kumar, M. Kundu, J. Landry, M. Laporte, W. Le, H. Y. Lei, M. J. Lenardo, B. Levine, A. Lieberman, K. L. Lim, F. C. Lin, W. Liou, L. F. Liu, G. López-Berestein, C. López-Otín, B. Lu, K. F. Macleod, W. Malorni, W. Martinet, K. Matsuoka, J. Mautner, A. J. Meijer, A. Meléndez, P. Michels, G. Miotto, W. P. Mistiaen, N. Mizushima, B. Mograbi, I. Monastyrska, M. N. Moore, P. I. Moreira, Y. Moriyasu, T. Motyl, C. Münz, L. O. Murphy, N. I. Naqvi, T. P. Neufeld, I. Nishino, R. A. Nixon, T. Noda, B. Nürnberg, M. Ogawa, N. L. Oleinick, L. J. Olsen, B. Ozpolat, S. Paglin, G. E. Palmer, I. Papassideri, M. Parkes, D. H. Perlmutter, G. Perry, M. Piacentini, R. Pinkas-Kramarski, M. Prescott, T. Proikas-Cezanne, N. Raben, A. Rami, F. Reggiori, B. Rohrer, D. C. Rubinsztein, K. M. Ryan, J. Sadoshima, H. Sakagami, Y. Sakai, M. Sandri, C. Sasakawa, M. Sass, C. Schneider, P. O. Seglen, O. Seleverstov, J. Settleman, J. J. Shacka, I. M. Shapiro, A. Sibirny, E. C. M. Silva-Zacarin, H. U. Simon, C. Simone, A. Simonsen, M. A. Smith, K. Spanel-Borowski, V. Srinivas, M. Steeves, H. Stenmark, P. E. Stromhaug, C. S. Subauste, S. Sugimoto, D. Sulzer, T. Suzuki, M. S. Swanson, I. Tabas, F. Takeshita, N. J. Talbot, Z. Tallóczy, K. Tanaka, K. Tanaka, I. Tanida, G. S. Taylor, J. P. Taylor, A. Terman, G. Tettamanti, C. B. Thompson, M. Thumm, A. M. Tolkovsky, S. A. Tooze, R. Truant, L. V. Tumanovska, Y. Uchiyama, T. Ueno, N. L. Uzcátegui, I. van der Klei, E. C. Vaquero, T. Vellai, M. W. Vogel, H. G. Wang, P. Webster, J. W. Wiley, Z. Xi, G. Xiao, J. Yahalom, J. M. Yang, G. Yap, X. M. Yin, T. Yoshimori, L. Yu, Z. Yue, M. Yuzaki, O. Zabirnyk, X. Zheng, X. Zhu and R. L. Deter, *Autophagy*, 2008, **4**, 151-175.

23 N. Mizushima and T. Yoshimori, *Autophagy*, 2007, **3**, 542-545.