Metallodrugs

Deutsche Ausgabe: DOI: 10.1002/ange.201509612
Internationale Ausgabe: DOI: 10.1002/anie.201509612

Anticancer Gold(III) Porphyrins Target Mitochondrial Chaperone Hsp60

*Di Hu[+], Yungen Liu[+], Yau-Tsz Lai, Ka-Chung Tong, Yi-Man Fung, Chun-Nam Lok, and Chi-Ming Che**

Abstract: *Identification of the molecular target(s) of anticancer metal complexes is a formidable challenge since most of them are unstable toward ligand exchange reaction(s) or biological reduction under physiological conditions. Gold(III) meso-tetraphenylporphyrin (**gold-1a**) is notable for its high stability in biological milieux and potent in vitro and in vivo anticancer activities. Herein, extensive chemical biology approaches employing photo-affinity labeling, click chemistry, chemical proteomics, cellular thermal shift, saturation-transfer difference NMR, protein fluorescence quenching, and protein chaperone assays were used to provide compelling evidence that heat-shock protein 60 (Hsp60), a mitochondrial chaperone and potential anticancer target, is a direct target of **gold-1a** in vitro and in cells. Structure–activity studies with a panel of non-porphyrin gold(III) complexes and other metalloporphyrins revealed that Hsp60 inhibition is specifically dependent on both the gold(III) ion and the porphyrin ligand.*

The distinct structural properties and reactivity conferred by metal–ligand coordination offer appealing possibilities for the design of new anticancer therapeutics based on metal coordination chemistry.[1] In this context, gold complexes have been increasingly studied since a number of examples have been reported to display promising activity towards various cancer cells and multidrug-resistant cancer cells in vitro.[2] Most reported examples of gold(III) complexes displaying antiproliferative properties have been noted to be unstable under physiological conditions, thus rendering subsequent development of therapeutic application a formidable challenge. In recent years, a number of gold(III) complexes supported by strong donor ligands have been reported to possess good stability and display potent anticancer activity,[3] and examples include a series of anticancer gold(III) porphyrins. Among them, [Au(TPP)]Cl (denoted **gold-1a**, Figure 1) is stable under physiological conditions, resistant to reduction by glutathione, highly cytotoxic to various cancer cell lines, including cisplatin-resistant cancer cells and multidrug-resistant cells, and exhibits potent in vivo anticancer properties in multiple animal tumor models.[4]

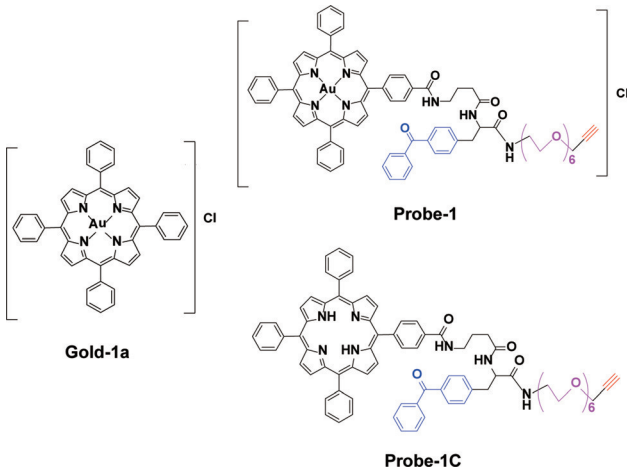


Figure 1. **Gold-1a**, clickable photoaffinity probe of **gold-1a** (probe-**1**), and a metal-free photoaffinity probe (probe-**1C**).

Identification of the direct molecular target(s) that account for the anticancer action of metal complexes has always been a difficult task. The outstanding stability of **gold-1a** under various biological conditions makes it possible to probe the molecular targets by using chemical biology approaches such as affinity-based proteome profiling. So far, there are only a few studies on protein target identification for metal compounds by using single affinity conjugates and pull-down techniques.[5] Of relevance to this work, photo-affinity and/or click chemistry probes of different types have been applied in studies of cisplatin-based compounds for analyzing Pt–DNA adducts and their interacting proteins.[6] In the present study, we utilized a clickable photo-affinity probe[7] to isolate the protein binding partner(s) of **gold-1a**. This analysis, together with various supporting biochemical and cellular experiments, revealed heat-shock protein 60 (Hsp60) to be an important molecular target of **gold-1a**. Hsp60 is an essential chaperone for mitochondrial protein transport and folding,[8] playing important roles in apoptosis regulation and tumor maintenance, and it is up-regulated in primary human cancerous tumors.[9] Only a few compounds that demonstrate in vitro inhibition of Hsp60 have been identified so far.[10] Herein, we show that gold(III) porphyrins target Hsp60 both in vitro and in cells.

Probe-**1** was synthesized by appending a linker (hexaethylene glycol), a clickable tag (alkyne), and a photo-affinity tag (benzophenone) onto one of the *meso*-phenyl rings of the porphyrin ligand of **gold-1a** (Figure 1). Another probe with a free *meso*-tetraphenylporphyrin ring, probe-**1C** (Figure 1), was also synthesized and used to examine non-specific

[*] D. Hu,[+] Dr. Y. Liu,[+] Dr. Y.-T. Lai, K.-C. Tong, Dr. Y.-M. Fung, Dr. C.-N. Lok, Prof. Dr. C.-M. Che
State Key Laboratory of Synthetic Chemistry, Chemical Biology Center, and Department of Chemistry, The University of Hong Kong
Pokfulam Road, Hong Kong (Hong Kong)
E-mail: cmche@hku.hk
[+] These authors contributed equally to this work.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201509612.

binding and specific binding owing to the presence of gold(III). Probe-**1** is cytotoxic to a number of cancer cell lines, with half-maximal inhibitory concentration (IC_{50}) values at low micromolar levels close to those of **gold-1a** (Table S1 in the Supporting Information). Probe-**1C** was not cytotoxic to the cancer cell lines examined at concentrations up to 100 μM.

The photo-affinity labeling of cellular proteins by probe-**1** was examined (Figure 2 and Figure S1 in the Supporting Information) by incubating cancer cells with probe-**1**, followed by UV irradiation and click reaction with azide-conjugated biotin. As shown in Figure 2A,B, a biotinylated protein of approximately 60 kDa was detected after incubating cells with probe-**1** at 1 μM for 1 h. The photo-affinity-labeled protein signals markedly diminished when cells were exposed to a 3-fold excess of **gold-1a**, thus suggesting that probe-**1** specifically recognizes the same protein target(s) as the parental **gold-1a**. No photo-affinity-labeled protein was detected for probe-**1C**, which comprises free H_2TPP ligand.

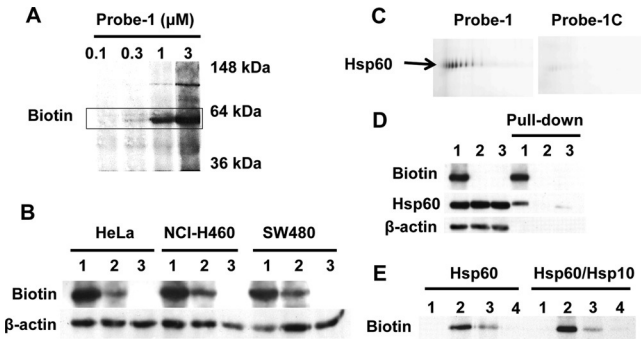


Figure 2. Identification of the protein labeled by probe-**1** as Hsp60. A) HeLa cells were treated with increasing concentrations of probe-**1** for 1 h followed by UV irradiation and click reaction with azide-conjugated biotin. The biotinylated proteins were detected by immunoblotting. B) HeLa, NCI-H460, and SW480 cells were incubated with probe-**1** (1), probe-**1** in the presence of 3-fold excess of **gold-1a** (2), and probe-**1C** (3). C) HeLa cells were treated with 4 μM probe-**1** or probe-**1C** followed by UV irradiation and click reaction with Cy5-azide. Isoformic protein spots labeled with Cy5 on a 2D gel were identified as Hsp60 by MALDI-TOF/TOF MS. D) HeLa cells were treated with 4 μM probe-**1** (1), probe-**1C** (2), or DMSO vehicle (3), followed by UV irradiation and click reaction with biotin-azide. The biotinylated proteins were isolated over streptavidin beads (pull-down) and detected by immunoblotting with Hsp60 antibody. E) Purified Hsp60 (0.5 μM) or Hsp60/Hsp10 complex (0.5 μM) were incubated with vehicle (1), 1 μM probe-**1** (2), 1 μM probe-**1** in the presence of a 10-fold excess of **gold-1a** (3), or 1 μM probe-**1C** (4), followed by photo-affinity labeling and click reaction with biotin-azide.

To identify the photo-affinity-labeled protein(s) of probe-**1**, the cell lysates were subjected to a click reaction with azide-conjugated Cy5, followed by resolution by two-dimensional gel electrophoresis (Figure 2C and Figure S2). Fluorescence scanning showed isoformic protein spots of around 60 kDa, which were identified as the heat-shock protein Hsp60 by MALDI-TOF/TOF MS (Table S2, SI). The photo-affinity-labeled proteins were also identified by a quantitative proteomics approach by using the stable isotope labeling by amino acids in cell culture (SILAC) technique followed by affinity isolation of biotinylated proteins and Orbitrap LC–MS/MS analysis (Figure S3).[7] Hsp60 was again identified as the photo-affinity-labeled protein, since it showed the highest enrichment ratio (Table S3). The identification of the photo-affinity-labeled protein as Hsp60 was also confirmed by biotin pull-down and immunoblot detection with an Hsp60 antibody (Figure 2D). Probe-**1** also directly labeled purified Hsp60, as well as Hsp60 in a complex with its cofactor, Hsp10, in vitro (Figure 2E). This labeling was diminished by the presence of a 10-fold excess of **gold-1a**. No significant photo-affinity labeling of Hsp60 was detected for probe-**1C** (Figure 2E).

The subcellular localization of **gold-1a** was examined by fluorescence imaging of cells treated with probe-**1**, followed by photo-affinity labeling and click chemistry with azide-conjugated fluorescein (FAM). Filamentous and punctate fluorescent signals that colocalized with a mitochondrial stain (MitoTracker Orange) and mitochondrial Hsp60 immunofluorescence were observed (Figure 3 and Figure S4). The mitochondria as a target of **gold-1a** was suggested by previous work.[4b]

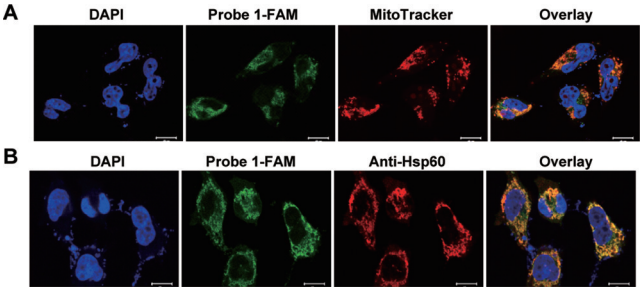


Figure 3. Fluorescence imaging of HeLa cells treated with 4 μM probe-**1** for 1 h, showing colocalization with the mitochondrial marker MitoTracker Orange (A) and Hsp60 immunofluorescence (B). Scale bar: 10 μm.

To assess whether Hsp60 is engaged by **gold-1a** inside cells, we applied a cellular thermal-shift assay (CETSA),[11] which is based on the biophysical principle of ligand-induced thermal stabilization of targeted proteins. Hsp60 in cells treated with **gold-1a** was significantly stabilized, as indicated by the marked temperature increases required for protein loss by denaturation/precipitation compared to untreated controls (Figure 4A). The thermal stability of Hsp60 increased as cells were treated with increasing concentrations of **gold-1a** (Figure 4B). Thus, the CETSA data provide evidence that Hsp60 is a molecular target that is engaged by **gold-1a** in cells. We also employed a previously described procedure to determine whether **gold-1a** impairs the chaperone activity of Hsp60 inside cells by measuring the activity of a mitochondrial substrate of Hsp60 (Figure S5).[12] The data are consistent with mitochondrial Hsp60 inhibition by **gold-1a**.

The direct impact of **gold-1a** on Hsp60 in vitro was investigated. The apparent binding of **gold-1a** to Hsp60 was demonstrated by saturable quenching of protein fluorescence by **gold-1a**, with a binding ratio of 1:1 and an apparent dissociation constant K_d of approximately 3.68 μM. Probe-**1** elicited very similar protein fluorescence quenching to **gold-**

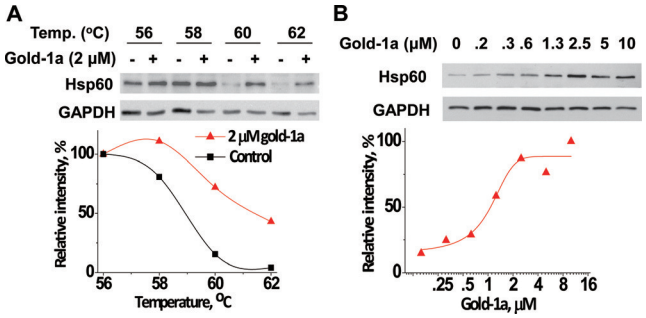
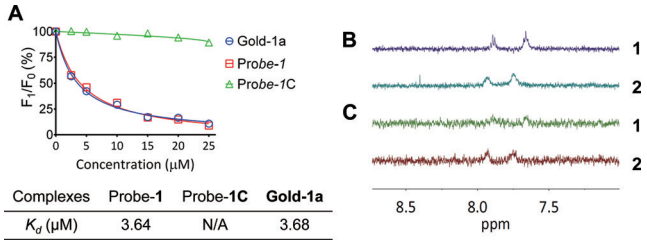


Figure 4. Cellular thermal-shift assay (CETSA). A) Stabilization of Hsp60 after incubating cells with **gold-1a**. The band intensity of soluble Hsp60 at 56 °C is set at 100%. B) Dose-dependent stabilization of Hsp60 by **gold-1a** in HeLa cells at a denaturing temperature (60 °C). The band intensity of Hsp60 with maximal stabilization is set to 100%.

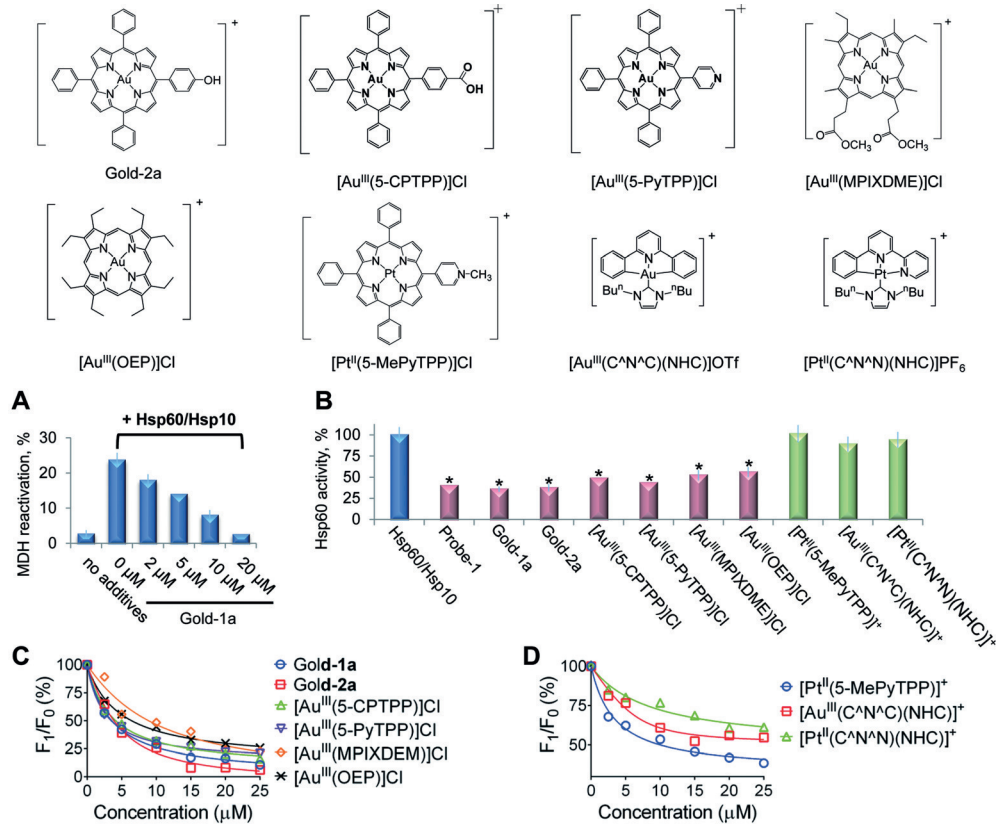


Complexes	Probe-**1**	Probe-**1C**	Gold-**1a**
K_d (μM)	3.64	N/A	3.68

Figure 5. The interactions of **gold-1a**, probe-**1**, and probe-**1C** with Hsp60 in vitro. A) Saturable quenching of Hsp60 protein fluorescence (19 μM; excitation: 262 nm; emission: 340 nm) by **gold-1a**, probe-**1**, and probe-**1C**, and the apparent dissociation constants (K_d). B) ^{1}H NMR spectra of **gold-1a** in the absence (1) or presence (2) of Hsp60. C) STD NMR spectra of **gold-1a** in the absence (1) or presence (2) of Hsp60.

1a but probe-**1C** did not have this effect (Figure 5A and Figure S6). The interaction between Hsp60 and **gold-1a** was further supported by saturation-transfer difference (STD) NMR measurements. In ^{1}H NMR, **gold-1a** shows signals at 7.5–8.0 ppm, attributable to protons in the phenyl ring and porphyrin. Both peak broadening and peak shifting were observed in the presence of Hsp60, thus confirming an interaction between **gold-1a** and Hsp60 (Figure 5B). Furthermore, the STD spectrum, which was only obtained from **gold-1a** in the presence of Hsp60, lends further support to the binding of **gold-1a** to Hsp60 (Figure 5C).

We next examined the effect of **gold-1a** on the chaperone activity of Hsp60 in the reactivation of denatured malate dehydrogenase (MDH) in vitro.[13] Hsp60/Hsp10 refolded denatured MDH to recover around 25% of the enzyme activity under standard assay conditions. When Hsp60/Hsp10 was treated with **gold-1a** (2–20 μM), there was a significant dose-dependent inhibition of the chaperone-mediated reactivation of denatured MDH (Figure 6A). Probe-**1** inhibited the chaperone activity of Hsp60 at a similar level to **gold-1a** (Figure 6B). It is noteworthy that **gold-1a** and all other complexes in the present study were tested for their effects on MDH activity and no inhibition was found.



E

	Cytotoxicity (IC_{50}, μM ± SD)						
	gold-1a	gold-2a	[Au^{III}(5-CPTPP)]Cl	[Au^{III}(5-PyTPP)]Cl	[Au^{III}(MPIXDME)]Cl	[Au^{III}(OEP)]Cl	[Pt^{II}(5-MePyTPP)]Cl
HeLa	2.87 ±0.17	2.62 ±0.07	5.58 ±0.22	1.07 ±0.01	3.17 ±0.04	3.40 ±0.11	>50.0

Figure 6. Structure–activity relationship of different porphyrin complexes with Hsp60. A) The dose-dependent effect of **gold-1a** on chaperone activity of Hsp60 in vitro in terms of MDH refolding. B) Effects of probe-**1**, gold(III) porphyrins, [Pt^{II}(5-MePyTPP)]$^+$, and non-porphyrin complexes (10 μM) on the chaperone activity of Hsp60. *$p \le 0.05$, $n \ge 3$. C, D) Quenching of Hsp60 (19 μM) fluorescence by gold(III) porphyrins (C) and others (D). E) In vitro cytotoxicity (24 h) of various gold(III) porphyrins and platinum(II) porphyrin against HeLa cells.

In addition to **gold-1a**, a series of gold(III) porphyrins (**gold-2a**,[14] [Au^{III}(5-CPTPP)]Cl, [Au^{III}(5-PyTPP)]Cl), [Au^{III}-(MPIXDME)]Cl, and [Au^{III}(OEP)]Cl)[15] were prepared (Figure 6). These gold(III) porphyrins significantly inhibited Hsp60 chaperone activity by more than 50% and strongly quenched Hsp60 fluorescence by more than 70% (Figure 6B, C). A Pt^{II} derivative of **gold-1a**, [Pt^{II}(5-MePyTPP)]Cl, which is mono-valent cationic owing to an N-substituted pyridine group, did not appreciably affect Hsp60 chaperone activity and fluorescence (Figure 6B, D). Two other non-porphyrin gold(III) and platinum(II) complexes, [Au^{III}-(C^N^C)(NHC)]OTf (H_2C^N^C = 2,6-diphenylphyridine; NHC = *N*-heterocyclic carbene)[16] and [Pt^{II}(C^N^N)-(NHC)]PF_6 (HC^N^N = 6-phenyl-2,2′-bipyridine),[17] did not significantly inhibit Hsp60 chaperone activity and only slightly quenched Hsp60 fluorescence (Figure 6B, D). Thus, while all of these metal complexes (except for [Pt^{II}(5-MePyTPP)]Cl) showed similar cytotoxicity towards HeLa cells, with low micromolar IC_{50} values (Figure 6E), only the gold(III) porphyrins generally display high binding activities and strong inhibitory effects on Hsp60.

The effects of some stable and fairly water-soluble metalloporphyrins were also examined. As shown in Figure S7, Ga^{III} protoporphyrin IX chloride (GaPPIX) markedly inhibited Hsp60 activity, while Sn^{IV} protoporphyrin IX dichloride (SnPPIX) showed modest inhibition and Zn^{II} protoporphyrin IX (ZnPPIX) had little effect. Free protoporphyrin IX (H_2PPIX) showed little effect on Hsp60. The mechanisms of inhibition of Hsp60 by the individual metalloporphyrins remain to be elucidated, but the specific roles of the metal ion centers are apparent.

The present study features the identification of a direct molecular target of stable anticancer metal complexes through the combination of a photo-affinity and click chemistry approach (the trifunctional clickable photo-affinity probe of **gold-1a**). Although there could be additional molecular targets that may escape detection with the present chemical probes, the mitochondrial chaperone Hsp60 as a direct molecular target of gold(III) porphyrins is supported by multiple independent assays. Structure–activity studies with a panel of non-porphyrin gold(III) and platinum(II) complexes, as well as other metalloporphyrins, revealed that the inhibitory activity on Hsp60 is specifically dependent on both the gold(III) ion and porphyrin ligand. It is tempting to speculate that the gold(III) porphyrins may bind to the target proteins through hydrophobic interactions with the porphyrin ligand as well as electrophilic interactions with the gold(III) ion. The gold(III) ion center is essential for Hsp60 inhibition, since platinum(II) and metal-free porphyrins failed to inhibit the activity of Hsp60. There are ample opportunities for optimizing the anticancer activities of gold(III) porphyrins by modifying the peripheral substituents on the porphyrin ligands and hence the interactions with Hsp60, as well as the cellular targeting.

Acknowledgements

This work was supported by the Innovation and Technology Fund (ITF-Tier 2 ITS/130/14FP) and a Special Equipment Grant of UGC (SEG_HKU02). We are indebted to Dr. K.-H. Sze and Dr. M.-K. Tse for their help with STD NMR experiment, Dr. W.-S. Lin for performing the cellular thermal-shift analysis, and Dr. T. Zou for constructive comments. We acknowledge the Centre for Genomic Sciences for mass spectrometric analysis and the Core Facility of Li Ka Shing Faculty of Medicine for confocal imaging.

Keywords: antitumor agents · biological targets · chemical proteomics · gold porphyrins · Hsp60

How to cite: *Angew. Chem. Int. Ed.* **2016**, *55*, 1387–1391
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Received: October 14, 2015
Published online: December 9, 2015

