VIP In Vivo Gold Chemistry

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Gold(III) Complexes Containing N-Heterocyclic Carbene Ligands: Thiol "Switch-on" Fluorescent Probes and Anti-Cancer Agents**

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Cellular thiol levels are linked to many diseases, including cancer and AIDS.[1] Soft metal ions and their complexes are usually sought after in the design of metal-based thiol probes and new anti-cancer drug leads that specifically target proteins or enzymes containing SH or SeH moieties.[2] Au^{I} is one of the soft metal ions with a high binding affinity towards thiol, and can be generated by the reduction of Au^{III}. Indeed, a majority of Au^{III} complexes are known to be readily reduced to Au^{I} or even Au^{0} by cellular thiol-containing compounds.[2a,3] As Au^{III} is usually 4-coordinated, whereas Au^{I} is usually 2-coordinated, the reduction of Au^{III} to Au^{I} is accompanied by the release of the coordinated ligand(s). Thus, we conceived that Au^{III} complexes containing strongly fluorescent organic ligand(s) could be potentially useful biological probes. The intraligand emission would be quenched owing to the presence of the low energy Au^{III} $5d_{x^2-y^2}$ orbital. Upon reduction of Au^{III} to Au^{I} by thiols, the fluorescent ligand(s) will be released, and hence emission would be switched on.

Gold(III) complexes are also known to be anti-cancer active and examples of this class of complexes that target proteins/enzymes and which can overcome cisplatin-related drug resistance can be found in the literature.[1e,3–4] Although the anti-cancer properties of many gold complexes, including that of Au^{III} and Au^{I}, are attributed to Au^{I},[3,4c,d,5] Au^{I} ions are unstable under physiological conditions.[2a] Recent work by others[5e,f,6] and our own studies have revealed that N-heterocyclic carbene (NHC) ligands are able to stabilize Au^{I} against reduction to Au^{0} and/or demetalation under physiological conditions.

Herein we report a series of cationic Au^{III} complexes containing both N^N^N and NHC ligands. The free H_2N^N^N ligands, 2,6-bis(imidazol-2-yl)pyridine (H_2IPI) and 2,6-bis(benzimidazol-2-yl)pyridine (H_2BPB), are highly emissive and display lower toxicity than 2,2′;6′,2″-terpyridine (terpy), and compounds bearing imidazolyl/benzimidazolyl groups have good water solubility and are widely used in biological studies.[7] The NHC ligand is incorporated to stabilize the potentially anti-cancer-active Au^{I} formed upon reduction of these Au^{III} complexes by physiological thiols. The Au^{III} complexes presented herein can act as fluorescent thiol "switch-on" probes, arising from the emission of the released N^N^N ligand upon reduction of Au^{III} to Au^{I}; one of these Au^{III} complexes has also been shown to suppress the growth of tumors in a nude mouse model.

The reaction of H_2IPI or H_2BPB with $KAuCl_4$ gave $[Au^{III}(N^N^N)Cl]$ (N^N^N = IPI, BPB). By refluxing a mixture of $[Au^{III}(N^N^N)Cl]$, silver triflate (AgOTf) and [Ag(NHC)X] (X = Br or I) in CH_3CN in the absence of light, complexes **1a–1e**, **2a–2b** (N^N^N = IPI) and **3a–3c** (N^N^N = BPB) were obtained (Figure 1). These Au^{III}–NHC complexes are soluble in CH_3OH, CH_3CN, DMF, and

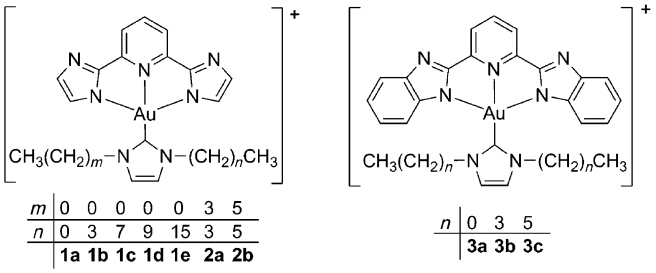

Figure 1. $[Au^{III}(N^N^N)(NHC)]OTf$ (OTf = trifluoromethane sulfonate) complexes **1** and **2** based on the IPI ligand, and **3** based on the BPB ligand.

DMSO. Complex **1a** shows a considerable water solubility (> 7 mg mL^{-1}); its ^{1}H NMR, ^{1}H–^{1}H COSY, and NOESY NMR spectra in D_2O are shown in the Supporting Information, Figure S1. Complexes **1d** and **1e** show amphiphilic properties. Changing both *N*-methyl groups of **1a** into longer alkyl groups (to form **2a** and **2b**), or the replacement of the imidazolyl groups with benzimidazolyl groups (to form **3a–3c**) renders the Au^{III}–NHC complex almost insoluble in water.

The structure of **1a** has been determined by X-ray crystallography (Figure S2, Table S1).[8] In its crystal structure, the molecules are paired through π–π interactions of the coordinated N^N^N ligands (interplane distance: ca. 3.3 Å). The NHC plane makes an angle of 64.9° with the N^N^N ligand plane, which is similar to the related angles in $[Au^{III}(C^N^C)(NHC)]^+$ (H_2C^N^C = 2,6-diphenylpyridine) (53.6–82.0°).[9] The Au^{III}–C_{NHC} distance of **1a** is 2.048 Å, which is slightly longer than those of $[Au^{III}(C^N^C)(NHC)]^+$

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(1.967–2.017 Å)[9] and $[Au^{III}(NHC)Cl_3]$ (1.975–2.013 Å),[10] but similar to those of $[Au^{III}(NHC)Br_3]$ (2.009–2.052 Å)[11] and *trans*-$[Au^{III}(NHC)_2I_2]BF_4$ (2.048 Å).[12]

Complexes **1**–**3** are stable in aqueous solutions. Taking **1a** or **3a** as an example (Figure S3), after 72 h no change in the UV/Vis absorption spectrum was found in PBS/DMSO (19:1, v/v, PBS = phosphate-buffered saline, 10 mM phosphate, pH 7.4, 298 K). Upon adding glutathione (GSH, 2 mM) to the solution, the absorption at 340–470 nm for **1a** and 370–460 nm for **3a** vanished within 1 min. ESI-MS analysis revealed the formation of free $H_2N^N^N$ ligand, $[Au^I(NHC)(GS)]$, and sulfur-bridged glutathione dimer (GSSG; Figure S4).

We employed ^1H NMR spectroscopy (400 MHz, in D_2O, pH* = 7.4, $[NaNO_3]$ = 0.1M, 298 K) to monitor the reaction of **1b** (0.83 mM) with GSH (5.83 mM). As shown in Figure 2a, the signals of the coordinated N^N^N ligand completely vanished, accompanied by the formation of free H_2IPI. The peaks at 3.31 are attributed to the β-CH_{cys} of GSSG;[13] the new peaks at 3.15 ppm and around 3.3 ppm are indicative of binding between GSH and Au^I, as similar chemical shifts attributed to β-CH_{cys} of $[Au^I(NHC)(Cys)]$ have been reported for the binding of cysteine with Au^I–NHC complexes.[6b] The signals of the NHC ligand are at 7.13 and 7.17 ppm, which is similar to those reported for $[Au^I(NHC)(Cys)]$ (7.15–7.30 ppm).[6b] Moreover, the ^1H NMR spectrum did not change upon increasing the reaction time from 10 min to 60 h (Figure S5), thus indicating the rapid completion of the reaction and the high stability of the products towards excess thiols. Similar results were found upon examination of the reactions of **1b** with GSH at molar ratios of 1:3 and 1:6 (Figure S6). As a result, one equivalent of the $[Au^{III}(N^N^N)(NHC)]^+$ complex reacted with three equivalents of GSH, resulting in the reduction of Au^{III} to Au^I and release of the N^N^N ligand. Two equivalents of GSH were oxidized to GSSG, and the remaining one equivalent of GSH coordinated to Au^I, giving $[Au^I(NHC)(GS)]$ (Figure 2b).

We also investigated the reaction of these Au^{III}–NHC complexes with thiol-free cellular reducing agent, ascorbic acid (2 mM). The absorption at 340–470 nm for **1a** did not change within 2 h, and that at 370–460 nm for **3a** started to notably decrease after 0.5 h. Furthermore, these absorptions did not disappear even after 24 h (Figure S7). These findings reveal that ascorbic acid is less effective than thiol in the reduction of Au^{III}.

The $[Au^{III}(N^N^N)(NHC)]^+$ complexes **1**–**3** are non-emissive in both the solid state and in solution. As described above, the reduction of Au^{III} to Au^I is accompanied by the release of the fluorescent N^N^N ligand. We found that once thiol-containing GSH or cysteine was added, predominant intraligand emission could be switched on within only 1 min, and the resultant emission spectra are the same as those of the free $H_2N^N^N$ ligands (Figure S8). Complexes containing IPI ligands, such as **1a** and **1e**, show emission of the released ligand mainly in the UV spectral region. Complex **3a**, which has a higher water solubility than **3b** and **3c**, and contains the highly fluorescent BPB ligand, which gives emission (as H_2BPB) in the visible spectral region (Figure 3a), was chosen for further study. It was found that the release of H_2BPB upon treatment of **3a** with GSH led to at least a 200-fold increase in emission intensity (Figure 3a); such a sensitivity towards thiol is much higher than that of the known Ru^{II} poly(1,10-phenanthroline)[14] and Cd^{II}-8-hydroxyquinoline-5-sulphonic acid[15] thiol probes. No fluorescence was found when **3a** was treated with Ca^{2+}, Mg^{2+}, or thiol-free amino acids such as Ser, Pro, Leu, Ile, His, or Ala. Complex **3a** is sensitive to thiol-containing compounds, including Cys, GSH, and the commonly used disulfide-bond reducing agent dithiothreitol (DTT; Figure S9a).

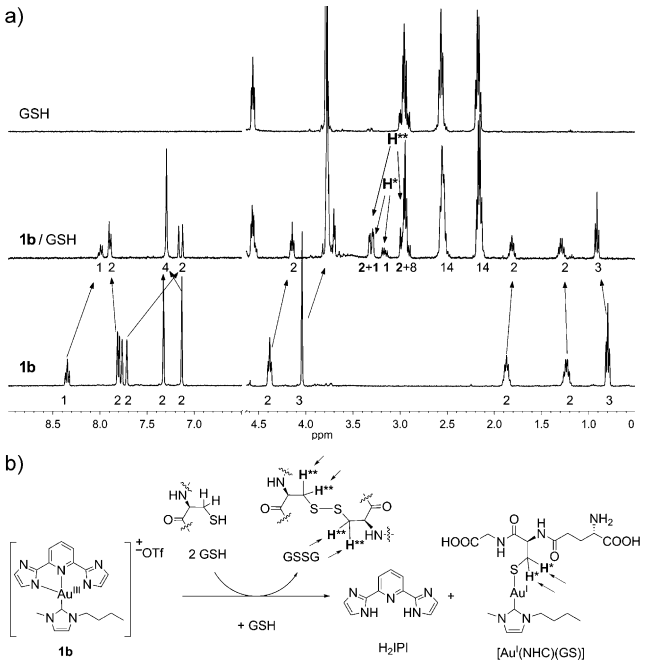

Figure 2. a) ^1H NMR spectra (400 MHz) of free GSH (top), a mixture of GSH and **1b** in a 7:1 molar ratio after mixing for 10 min (middle), and **1b** (bottom); intensity ratios are shown. b) Reaction of **1b** with GSH under near-physiological conditions.

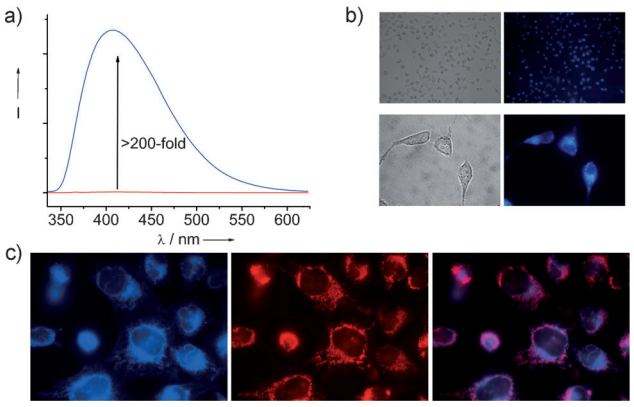

Figure 3. a) Fluorescence intensity (I) before (——) and after (——) adding GSH (2 mM) to **3a** (20 μM) in PBS containing 10% DMSO (v/v). b) Fluorescence microscopy images (upper: 10× and lower: 40×) of HeLa cells treated with 20 μM **3a** for 10 min. Images were taken without fixing or washing the cells. c) Fluorescence images of **3a** (left, 365 nm excitation), mitochondria-specific Mito-tracker Red stain (middle, 546 nm excitation), and the merged image (right).

We further studied the sensitivity of the **3a** probe (20 μM) to thiol-containing human serum albumin (HSA) at a concentration of 5 mg mL^{-1} (75 μM). As shown in Figure S9b, the emission intensity increased slowly and started to level off after 120 min, and the maximal response was only 20% compared to that towards GSH under equivalent conditions. Each HSA molecule contains only one free thiol group, Cys34, and Cys34 is surrounded by side chains of Pro35, Asp38, His39, Val77, and Tyr84;[16] these side chains may hamper direct interaction of the free thiol group with the Au^{III}–NHC complexes.

In view of the favorable fluorescence switch-on property of **3a** towards low-molecular-weight thiols, we investigated the uptake and intracellular distribution of **3a** in cancer cells by fluorescence microscope. After incubating human cervical epithelioid carcinoma (HeLa) cells with **3a** (20 μM in minimum essential medium with 10% fetal bovine serum, v/v) for only 10 min, significant blue fluorescence was detected in the cytoplasm, but not in the nucleus or extracellular environment (Figure 3b); this is suggestive of the stability of **3a** towards cell culture media and the efficient cellular uptake and transformation of Au^{III}–NHC complex into Au^{I}–NHC in the cytoplasm. The specific cellular location of the fluorescence was further studied by co-localization analysis with organelle-specific stains. It was found that a significant portion of the fluorescence signal in the **3a**-treated cells was localized in the mitochondria, and could be specifically stained with Mito-tracker Red (Pearson's correlation coefficient $R = 0.74$; Figure 3c). Notably, this result indicates cellular uptake of **3a** and intracellular reduction took place within 10 min. As mentioned above, the reduction of **3a** by ascorbic acid became notable only after 0.5 h and the reaction of **3a** with large-molecular-weight serum albumin was much slower, with only 20% reaction observed after a reaction time of 120 min. Thus, ascorbic acid and serum albumin would not pose a hurdle for **3a** entering cancer cells, for tracing the intracellular thiols, or for delivering the [Au(NHC)]$^+$ moiety to thioredoxin reductase (TrxR), as described below.

On the basis of the above findings, the Au^{III}–NHC complexes **1**–**3** could be reduced by intracellular GSH to give Au^{I}–NHC complexes. This reaction is reminiscent of the activation process of Pt^{IV} prodrugs, in which Pt^{IV} is reduced to Pt^{II} through intracellular reduction by GSH, with concomitant GSSG formation.[17] Thus, in a cellular pharmacological context, these Au^{III}–NHC complexes can be conceived of as precursors of anti-cancer Au^{I}–NHCs complexes. Berners-Price, Filipovska, and co-workers[6b] demonstrated that Au^{I}–NHCs complexes could induce apoptosis of cancer cells, presumably through the inhibition of TrxR, a thiol- and selenol-containing flavoenzyme that reduces thioredoxin and plays a key role in cancer cell progression.[3b] Our MTT assays showed that the Au^{III}–NHC complexes inhibited the growth of different cancer cell lines, including HeLa, hepatocellular carcinoma (HepG2), breast cancer (MCF-7), non-small-cell lung carcinoma (NCI-H460), nasopharyngeal carcinoma (SUNE1), and mouse melanoma (B16), with IC_{50} values ranging from 1.4 ± 0.2 to 55.0 ± 8.5 μM (Table S2). The cytotoxicity of these Au^{III} complexes increases with increasing lipophilicity (Figure S10). The free $H_2N^N^N$ ligand did not exert significant cytotoxicity, even at a concentration of 100 μM (Figure S11). The cytotoxicity of **3a** was investigated in the context of the expression of apoptotic proteins using western blot analysis. After treatment of HeLa cells with **3a** (15 μM) for 48 h, cleaved apoptotic caspases 3, 7, and 9, and poly(ADP-ribose) polymerase (PARP) were activated (Figure S12), which reveals that the **3a**-treatment induced apoptotic cell death.

The effects of the Au^{III}–NHC complexes on cellular TrxR were estimated using 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) as a substrate. Treatment of HeLa cells with **1a**, **1b**, **1e**, or **3a** for 1 h resulted in significant inhibition of the TrxR activity in the cell lysates, with IC_{50} values of 14.3, 7.7, 3.8, and 5.5 μM, respectively (Table 1). The cytotoxicity IC_{50} values for 72 h treatment with **1a**, **1b**, **1e** and **3a** were 32.9, 16.5, 1.4 and 14.4 μM, respectively (Table 1). Thus, the inhibitory potency of these complexes on TrxR could be reasonably correlated with their cytotoxicity. Notably, the 72 h cytotoxicity IC_{50} of **1e** (1.4 μM) is comparable to that of auranofin (1.8 μM), a potent TrxR inhibitor that can induce apoptotic cell death.[3b] The weaker inhibition of the enzyme activity by **1a**, **1b**, **1e**, and **3a** compared to that by auranofin may be due to the lower cellular uptake rate of gold for these [Au^{III}(N^N^N)(NHC)]$^+$ complexes (Table 1), which is probably caused by their hydrophilic nature. As TrxR is an important biomolecular target of Au^{I}–NHC and is abundant in mitochondria as well as in cytoplasm,[6h] the specific cytoplasmic localization according to the above-mentioned findings with a fluorescence microscope is supportive of **3a** delivering anti-cancer-active Au^{I}–NHC to the bio-molecular target.

Table 1: Cytotoxicity IC_{50} [μM], TrxR inhibition IC_{50} [μM], cellular uptake [μg g^{-1}], and lipophilicity of **1a**, **1b**, **1e**, **3a**, and auranofin.

Entry	Compound	Cytotoxicity[a]	TrxR[b]	Uptake[c]	log *P*[d]
1	**1a**	32.9 ± 1.3	14.3 ± 2.0	12.3 ± 1.1	−1.41
2	**1b**	16.5 ± 1.4	7.7 ± 1.6	22.3 ± 2.1	−0.93
3	**1e**	1.4 ± 0.2	3.8 ± 0.5	129.9 ± 11.0	2.35
4	**3a**	14.4 ± 2.2	5.5 ± 0.3	12.1 ± 0.7	−0.05
5	auranofin	1.8 ± 0.1	0.57 ± 0.06	1189.3 ± 85.6	nd

[a] Cytotoxicity towards HeLa cells was determined by MTT assay. [b] TrxR activity of HeLa cell lysates was estimated using DTNB as a substrate in the presence of NADPH. [c] Cellular uptake was determined by the gold content (in μg) in the cell proteins (in g) after treating HeLa cells with each complex for 10 min. [d] Lipophilicity was determined by measuring λ_{max} for each complex in *n*-octanol and water containing sodium chloride (0.9% w/v). nd = not determined.

We further tested the in vivo tumor inhibition effects of the most potent **1e**. After treatment of nude mice bearing HeLa xenografts with 3 mg kg^{-1} of **1e** three times per week, a significant reduction in tumor volume was found after 11 ($p < 0.05$) and 14 ($p < 0.01$) days, with tumor inhibition of up to 60% and 76%, respectively (Figure 4a,b). No mouse death or body weight loss was found (Figure S13).

In conclusion, a class of Au^{III} complexes bearing N-heterocyclic carbene and 2,6-bis(imidazol-2-yl)pyridine or 2,6-bis(benzimidazol-2-yl)pyridine ligands have been developed. These Au^{III}–NHC complexes are sensitive towards thiols, which leads to release of the fluorescent $H_2N^N^N$

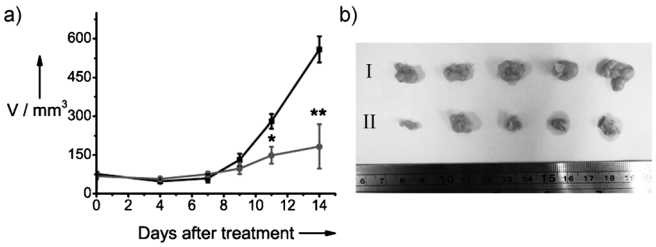

Figure 4. a) Average tumor volumes (V) of mice bearing HeLa xenografts after treatment with **1e** (3 mg kg^{-1}; ——●——) or solvent (——■——) through intratumoral injection. b) Photographs of mouse tumors in different groups after treating for 14 days. I, solvent control; II, **1e** (3 mg kg^{-1}). * = $p < 0.05$, ** = $p < 0.01$ compared to solvent control.

ligand, and thus can serve as a switch-on probe for thiols in biological systems. The Au^{III} complexes, through the formation of Au^{I}–NHCs upon Au^{III} reduction, can also suppress tumor growth in mice bearing HeLa xenografts. As the emission properties of the released $H_2N^N^N$ ligand and the reduction of Au^{III} to Au^{I} can be modulated and systematically tuned by ligand modification, these Au^{III}–NHC complexes could be a promising scaffold for the future development of novel switch-on bio-probes and anti-cancer agents.

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[8] CCDC 914048 (**1a**) contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

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