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# Modification of HIV-1 reverse transcriptase and integrase activity by gold(III) complexes in direct biochemical assays

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ABSTRACT

Gold(I) and gold(III) complexes have been previously investigated for potential biomedical applications including as anti-HIV agents. The oxidising nature of some gold(III) complexes yields well-documented cellular toxicity in cell-based assays but the effect in direct biochemical assays has not been fully investigated. In this study, gold(III) complexes were evaluated in HIV-1 reverse transcriptase and HIV-1 integrase biochemical assays. The gold(III) tetrachlorides $KAuCl_4$ and $HAuCl_4$ yielded sub-micromolar $IC_{50}$'s of 0.947 and 0.983 μM in the direct HIV-1 RT assay, respectively, while $IC_{50}$'s ranging from 0.461 to 8.796 μM were obtained for seven selected gold(III) complexes. The gold(III) tetrachlorides were also effective inhibitors of integrase enzymatic activity with >80% inhibition obtained at a single dose evaluation of 10 μM. RT inhibition was decreased in the presence of a reducing agent (10 mM DTT) and against the M184V HIV-1 RT mutant, while none of the gold(III) complexes were effective inhibitors in cell-based antiviral assays (SI values <5.95). Taken together, the findings of this study demonstrate that gold(III) complexes modify HIV-1 enzyme activity in direct biochemical assays, most likely through protein oxidation.



## 1. Introduction

Gold compounds have found clinical relevance as disease-modifying anti-rheumatic drugs (DMARD's) in the form of Auranofin (Riduara™), aurothioglucose (Solganol™) and aurothiomalate (Myochrisine™ or Aurolate™).[1] The success of the platinum-containing cisplatin and carboplatin also stimulated an interest in gold-based anti-cancer agents which has yielded extensive scientific research into this field.[2–12] To a lesser extent, gold-compounds have also been evaluated as antimicrobial, anti-malarial[13] and anti-HIV agents.[14–21] Although an exact mechanism of anti-HIV activity is yet to be elucidated, gold-based compounds have demonstrated effective antiviral activity in both cell-based[15–19] and direct enzymatic assays[18–21]; in particular, reverse transcriptase (RT)[18–21] and protease[21] biochemical assays. Okada and co-workers[18] detailed the anti-RT activity of bis(thioglucose)gold(I) while in the study by Sun and co-workers,[20] the authors demonstrated the nanomolar inhibition of RT activity by several gold(III) porphryins. In Fonteh et al., 2009,[21] the authors described the direct inhibition of both RT and protease enzyme activity by a tetrachloro-(bis-(3,5-dmintehylpyrazol)methane) gold(III) chloride.

Although stable in aqueous media, gold(III) is readily reduced by biologically occurring reductants, such as thiols, to the more stable gold(I) oxidation state.[1] In cell-based assays, gold(III) complexes can act as strong oxidising agents, leading to the well-documented cellular toxicity intrinsic to this class of compounds. The effect of the oxidation ability of gold(III) complexes in direct enzymatic assays has not been fully investigated; however, some published work has attributed the inhibitory activity of gold(III) complexes in direct assays to protein/enzyme oxidation.[22,23] Here, we demonstrate that gold(III) complexes may be potent oxidisers of HIV-1 RT and integrase enzymes and may impact the outcome of direct biochemical assays. The oxidising nature of gold(III) needs to be taken into account in these assays and potentially in other direct biochemical assays as well.

## 2. Materials and methods

### 2.1. Recombinant enzymes, viral isolates, molecular clones, inhibitors, and test compounds

The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID,

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NIH: HIV-$1_{HXB2}$ Reverse Transcriptase/MI84V from Dr. Vinayaka Prasadand (catalogue number 3195); HIV-$1_{HXB2}$ Reverse Transcriptase ($p66^{E>Q}$/p51) from Dr. Stuart Le Grice (catalogue number 2897); HIV-1 Ba-L from Dr. Susanne Gartner, Dr. Mikulas Popovic and Dr. Robert Gallo (catalogue number 510); HIV-1 NL4-3 molecular clone from Dr. Malcolm Martin (catalogue number 114); and Nevirapine (catalogue number 4666). AZT-tp was obtained from GeneCraft, Germany while Auranofin was obtained from Sigma, RSA. $HAuCl_4$, $KAuCl_4$ and $Au(dppe)_2Cl$ were synthesised in house by standard methods.

$[Au(bipy^R)(OH)_2][PF_6]$ (**1**–**2**) and $[(bipy^R)_2Au_2(\mu\text{-}O)_2][PF_6]_2$ (**3**–**4**) were prepared according to Cinellu et al., 2000[24]; *cis*-$[Au(pz^{Ph}\text{-}H)(PPh_3)Cl_2]$ (**5**) and *trans*-$[Au(pz^{Ph}\text{-}H)(py^{2Me})Cl_2]$ (**6**) according to Minghetti et al., 1998[25]; $[Au(py^{dmb}\text{-}H)(OAc)_2]$ (**7**) was prepared by standard methods starting from $[Au(py^{dmb}\text{-}H)Cl_2]$[26] (vide infra). Elemental analyses were performed with a Perkin-Elmer Elemental Analyzer 240B by Mr. A. Canu (Dipartimento di Chimica, Università di Sassari, Italy). Conductivity measurements were performed with a Philips PW 9505 conductivity meter. Infrared spectra were recorded with a FT-IR Jasco 480P instrument using Nujol mulls. $^1H$ and $^{31}P\{^1H\}$ NMR spectra were recorded at 293 K with a Varian VXR 300 spectrometer operating at 300.0 and 121.4 MHz, respectively; chemical shifts are given in ppm relative to internal TMS ($^1H$) and external $H_3PO_4$ ($^{31}P$) signals. Mass spectra were recorded with a VG 7070 instrument operating under FAB conditions, with 3-nitrobenzyl alcohol as supporting matrix.

### 2.2. Analytical and spectroscopic data of complexes 1–7

*For* ***1****:* Yield = 80%; mp 192 °C; Elemental Anal. Calcd for $C_{10}H_{10}AuF_6N_2O_2P$: C, 22.57; H, 1.89; N, 5.26; found: C, 22.65; H, 1.78; N, 5.03; FAB-MS: *m*/*z* 387 $[Au(bpy)(OH)_2^+]$; $\Lambda_M(5\times10^{-4}$ M, $Me_2CO)$ 158 $\Omega^{-1}$ cm$^2$ mol$^{-1}$; IR (Nujol, cm$^{-1}$): *ν* 3499 m (broad) (O–H), 2375 w (broad) (O–H···O), 1604 s, 840 vs ($PF_6$); $^1H$ NMR 300 MHz (acetone-$d_6$): *δ* 4.15 (s, 2H, OH), 8.25 (ddd, *J* = 8.1, 5.9, 1.5 Hz, 2H, $H^{5,5'}$), 8.76 (td, *J* = 7.8, 1.5 Hz, 2H, $H^{4,4'}$), 8.92 (d, *J* = 8.1 Hz, 2H, $H^{3,3'}$), 9.14 (d, *J* = 5.9 Hz, 2H, $H^{6,6'}$).

*For* ***2****:* Yield = 65%; mp 179 °C; Elemental Anal. Calcd for $C_{18}H_{26}AuF_6N_2O_2P$: C, 33.55; H, 4.07; N, 4.35; found: C, 33.65; H, 4.02; N, 4.33; $\Lambda_M(5\times10^{-4}$ M, $Me_2CO)$ 150 $\Omega^{-1}$ cm$^2$ mol$^{-1}$; IR (Nujol, cm$^{-1}$): *ν* 3600 m, 3530 m, 2370 w, 1615 s, 1540 m, 1085 m, 834 vs; $^1H$ NMR 300 MHz ($CD_3CN$): *δ* 1.50 (s, 18H, $CH_3$), 3.21 (s, 2H, OH), 7.99 (dd, *J* = 6.2, 2.0 Hz, 2 H, $H^{5,5'}$), 8.54 (d, *J* = 2.0 Hz, 2H, $H^{3,3'}$) 9.12 (d, *J* = 6.2 Hz, 2H, $H^{6,6'}$).

*For* ***3****:* Yield = 45%; mp 186 °C; Elemental Anal. Calcd for $C_{20}H_{16}Au_2F_{12}N_4O_2P_2$: C, 23.36; H, 1.57; N, 5.45; found: C, 23.11; H, 1.30; N, 5.38; FAB-MS: *m*/*z* 739 $[[Au(bipy)(\mu-O)_2^{2+}]+H]$; $\Lambda_M(5\times10^{-4}$ M, MeCN) 240 $\Omega^{-1}$ cm$^2$ mol$^{-1}$; IR (Nujol, cm$^{-1}$): *ν* 1605 vs, 1564 w, 1498 m, 838 vs ($PF_6$), 682 s and 662 s ($Au_2O_2$); $^1H$ NMR 300 MHz ($CD_3CN$): *δ* 8.08 (td, *J* = 5.9, 3.2 Hz, 2H, $H^{5,5'}$), 8.49–8.56 (m, 4H, $H^{3,3'}$+$H^{4,4'}$), 8.66 (dt, *J* = 5.6, 1.1 Hz, 2H, $H^{6,6'}$).

*For* ***4****:* Yield = 56%; mp 217 °C; Elemental Anal. Calcd for $C_{36}H_{48}Au_2F_{12}N_4O_2P_2$: C, 34.52; H, 3.86; N, 4.47; found: C, 34.33; H, 3.78; N, 5.4.36; $\Lambda_M(5\times10^{-4}$ M, MeCN) 230 $\Omega^{-1}$ cm$^2$ mol$^{-1}$; IR (Nujol, cm$^{-1}$): *ν* 1615 s, 1545 m, 840 vs, 680 s and 655 s ($Au_2O_2$); $^1H$ NMR 300 MHz ($CD_3CN$): *δ* 1.51 (s, 18H, $CH_3$), 8.02 (dd, *J* = 6.2, 2.0 Hz, 2H, $H^{5,5'}$), 8.51 (d, *J* = 6.2 Hz, 2H, $H^{6,6'}$), 8.52 (d, *J* = 2.0 Hz, 2H, $H^{3,3'}$).

*For* ***5****:* Yield 72%; mp 118 °C; Elemental Anal. Calcd for $C_{27}H_{22}PAuCl_2N_2$: C, 48.16; H, 3.29; N, 4.16; found: C, 48.88; H, 3.44; N, 4.35; FAB-MS: *m*/*z* 673 $[M^+]$; $\Lambda_M$ ($6.2\times10^{-4}$ M, dichloromethane): 6.45 $\Omega^{-1}$ cm$^2$ mol$^{-1}$; IR (Nujol, cm$^{-1}$): *ν* 1598 m, 1100 s, 690 vs, 319 vs, 302 s; $^1H$ NMR 300 MHz ($CDCl_3$): *δ* 7.07 (s, 1H, $H^3$/$H^5$ pz), 7.08 (s, 1H, $H^5$/$H^3$ pz), 7.22–7.76 (m, 20H, $C_6H_5$-N(1)+P($C_6H_5$)$_3$); $^{31}P$ NMR ($CDCl_3$): *δ* 34.3.

*For* ***6****:* Yield 51%; mp 184 °C; Elemental Anal Calcd for $C_{16}H_{16}AuCl_2N_3$: C, 37.08; H, 3.11; N, 8.11; found: C, 37.14; H, 3.29; N, 7.77; IR (Nujol, cm$^{-1}$): *ν* 1593 s, 688 s, 368 s; FAB-MS: *m*/*z* 517 $[M^+]$; $^1H$ NMR 300 MHz ($CDCl_3$): *δ* 2.45 (s, 6H, $CH_3$); 7.27 (t, *J* = 7.4 Hz, 1H, $H^4$ Ph); 7.45 (t, *J* = 7.7 Hz, 2H, $H^{3,5}$ Ph); 7.63 (s br, 1H, $H^4$ $py^{2Me}$); 7.71 (d, *J* = 7.8 Hz, 2H, $H^{2,6}$ Ph); 7.86 (s, 1H, $H^3$/$H^5$ pz), 8.05 (s, 1H, $H^5$/$H^3$ pz); 8.56 (s br, 2H, $H^{2,6}$ $py^{2Me}$).

$[Au(py^{dmb}\text{-}H)(OAc)_2]$ (**7**): Solid AcOAg (0.170 g, 1.02 mmol) was added to a solution of $[Au(py^{dmb}\text{-}H)Cl_2]$ (0.233 g, 0.5 mmol) in acetone (15 mL). The resulting suspension was stirred at room temperature for 8 h and then filtered through Celite. Addition of diethyl ether to the concentrated solution afforded a creamy solid product. Recrystallization from chloroform/diethyl ether gave the analytical sample. Yield 70%; mp 201 °C (dec); Elemental Anal Calcd for $C_{18}H_{20}AuNO_4$: C, 42.28; H, 3.94; N, 2.74; found: C, 42.08; H, 3.70; N, 2.75; IR (Nujol, cm$^{-1}$): *ν* 1677 vs, 1636 vs, 1604 s, 1300 vs, 1281 vs, 1033 m, 1013 m, 778 s, 754 s, 680 s; FAB-MS: *m*/*z* 452 $[M-CH_3CO_2]$; $^1H$ NMR 300 MHz ($CDCl_3$): *δ* 2.08 (s, $CH_3$), 2.09 (s, $CH_3$), 2.15 (s, $CH_3CO_2$), 2.46 (s br, $CH_3CO_2$), 7.02–7.06 (m, 1H), 7.17–7.20 (m, 2H), 7.38 (dd, *J* = 8.1, 1.0 Hz, 1H), 7.43 (td, *J* = 5.9, 1.5 Hz, 1H), 7.82 (d, *J* = 7.1 Hz, 1H), 8.03 (td, *J* = 7.8, 1.7 Hz, 1H), 9.14 (dd, *J* = 5.9, 1.5 Hz, 1H).

### 2.3. Direct enzymatic assays

The gold-based compounds ($HAuCl_4$, $KAuCl_4$, Auranofin, $Au(dppe)_2Cl$, **1–7**) AZT-tp, and Nevirapine were evaluated for the direct inhibition of reverse transcriptase activity using a commercially available assay (Roche Diagnostics, Germany) and recombinant reverse transcriptase (wild type, M184V mutant,[27] and $p66^{E>Q}$/p51 mutant[28]). Briefly, the test compounds were prepared and evaluated at a single-dose concentration of 10 μM in the absence or presence of 10 mM dithiothreitol (DTT, Sigma, RSA) to determine a percentage inhibition or at 8 different concentrations, ranging from 20 μM to 50 nM, to determine a dose–response curve and $IC_{50}$ value. The experiments were conducted according to manufacturer's instructions and the colorimetric endpoint was measured on a microplate reader (xMark, Bio-Rad, USA) at 405 nm with a reference wavelength of 490 nm. Each experiment was conducted in triplicate and $IC_{50}$ values were determined using Origin 6.1 Software (Origin Lab Corporation, USA).

The HIV-1 integrase strand-transfer inhibition assay used in this study was adapted from previously described methods.[29] Briefly, a 30-bp double-stranded biotinylated donor DNA, corresponding to the HIV U5 viral DNA end, was added to the wells of streptavidin-coated 96-well microtiter plates. Following one hour incubation at room temperature and a stringent wash step, purified recombinant integrase was assembled onto the pre-processed donor DNA through incubation for 30 min at 22 °C. Following another wash step, the gold tetrachlorides ($HAuCl_4$ and $KAuCl_4$) were titrated into individual wells at a single dose concentration of 10 μM. The microtiter plates were incubated for 30 min at 37 °C, washed and the strand transfer reaction was initiated through the addition of FITC-labelled target DNA. After an incubation period, the plates were washed as before and an AP-conjugated anti-FITC secondary antibody was added. Finally, the plates were washed and substrate was added to allow for photometric measurement at 620 nm using a microplate reader (xMark, Biorad, USA). Each experiment was conducted at least in triplicate.

### 2.4. Cell-based assays

Fresh human PBMCS were isolated from the blood of HIV- and HBV-seronegative donors (Biological Speciality Corporation, Colmar, PA, USA) by means of Ficol-Hypaque density gradient separa-

tion and PHA-stimulated for 48–72 h at 37 °C, followed by the addition of IL-2 to maintain stimulation. PBMC's from at least two different donors were pooled and plated in a 96-well round bottomed microtiter plate at $5 \times 10^4$ cells/mL. Gold(III) complexes (**1–7**) were prepared at 2× concentration and 100 μL of each concentration was added to the appropriate wells. For the antiviral assays, 50 μL of virus (HIV-1 Ba-L; Subtype B, CCR5-tropic, isolated from primary culture of adherent cells grown from explanted human infant lung tissue,[30,31] and HIV-1 NL4-3; Subtype B, CXCR4-tropic, molecular clone[32]) at 0.1 MOI was added to each well for a total volume of 200 μL/well. Separate plates were prepared identically without virus for evaluation of compound cytotoxicity. Each plate was incubated at 37 °C and 5% $CO_2$ for 7 days.

To determine cytotoxicity of the compounds, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenol)-2-(4-sulfophenyl)-2H-tetrazolium (MTS, CellTiter 96 Reagents, Promega, USA; 25 μL/well) was added to the microtiter plates without virus. Following an incubation period of 4 h at 37 °C and 5% $CO_2$, the plates were read at an absorbance of 490 nm with a reference wavelength of 650 nm using a SPECTRAmax plate reader (Molecular Devices, USA). The cytotoxic concentration ($CC_{50}$) values were determined as the concentration of the compound required to reduce the cell viability of cells by 50%.

For the antiviral assays, cell-free supernatant samples were collected and analysed for reverse transcriptase activity according to a previously described procedure.[33] The $EC_{50}$ values were calculated as the compound concentration required to reduce HIV-1 replication by 50% while the selectivity index (SI) values were calculated as the ratio of $CC_{50}/EC_{50}$.

### 2.5. NMR analysis

NMR spectra were recorded at 298 K with a 400 MHz Bruker Avance Spectrometer equipped with a BBI 5 mm probe. NMR were obtained from STD off resonance spectra using the stdwsrf.ak pulse program (Avance version 00/02/07). Water suppression watergate was achieved by a W5 pulse sequence with gradients using double echo.[34] NMR samples were prepared in phosphate-buffered saline (PBS) in $D_2O$, from a stock solution of gold(III) compound in dmso-$d_6$. Proton chemical shifts were referenced internally to residual solvent resonances and quoted in ppm.

### 2.6. Computational modelling

All virtual protein manipulations were performed on a dual-core desktop PC with a Red Hat Enterprise Linux Version 5.0 operating system, using the commercial software package SybylTM version 8.0, licensed from TriposTM (Tripos Inc., St. Louis, MO, USA, 2008). All protein crystal structures used in the virtual manipulations were retrieved from the protein data bank (PDB). Specifically, the p66 subunit of HIV-1 RT (PDB code: 2won) was used to highlight the positions of the cysteine (Cys) and methionine (Met) residues in relation to the inhibitor binding site (with bound nevirapine) and is represented in Figure 2a. The HIV-1 IN monomer model illustrated in Figure 2b was constructed from various crystal structures of the enzyme subdomains: the catalytic core domain (PDB code: 1qs4), the C-terminal domain (PDB code: 1ex4) and the N-terminal domain (PDB code: 1k6y) were connected and the missing loop sequence between the catalytic core and N-terminal domains (residues 141–144) completed according to the 2008 Los Alamos consensus sequence[35] for HIV-1 subtype B integrase using the Biopolymer function in the Sybyl suite of programs. The structure was minimised with no initial optimisation using the AMBER7FF99 force field (5000 iterations). All cysteine and methionine residues in the HIV-1 IN monomer model were rendered in CPK to illustrate their positions relative to each other and to the enzyme active site.

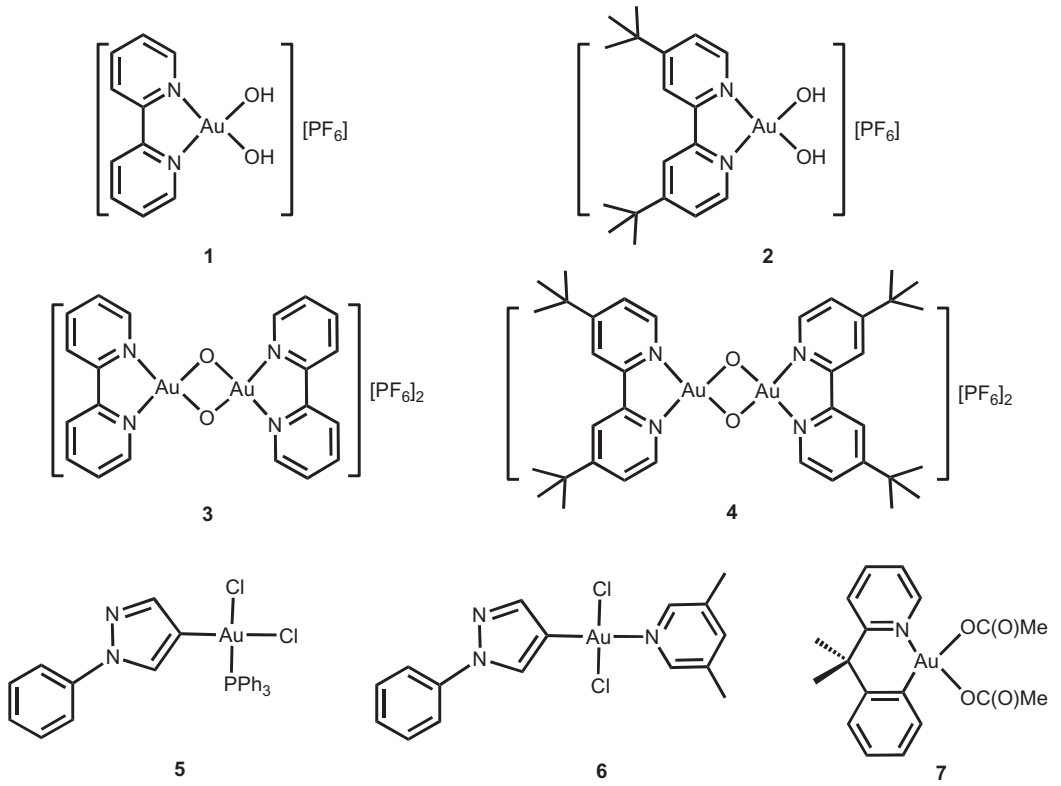


**Figure 1.** Schematic representation of the seven gold(III) compounds identified as having inhibitory activity towards HIV-1 reverse transcriptase in direct enzyme assays. Compounds **1–4** are coordination complexes while compounds **5–7** are organometallic compounds.

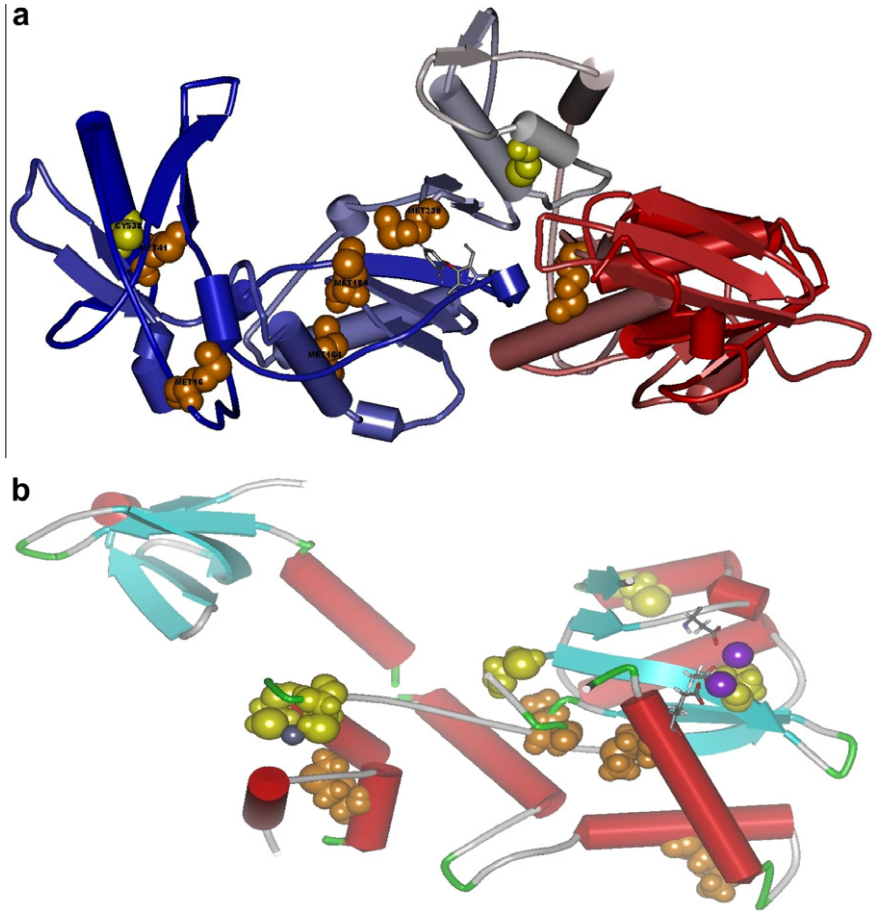


**Figure 2.** Schematic models of (a) the p66 domain of HIV-1 RT detailing the cysteinyl residues (in yellow) at positions 280 and 38 as well as the positions of the six methionine residues (in orange) and (b) the full length HIV-1 integrase monomer with N-terminal domain, C-terminal domain and central catalytic domain (CCD) detailing the positions of the five cysteine (in yellow) and four methionine residues (in orange).

## 3. Results and discussion

### 3.1. Chemistry

The compounds selected for this study belong to two different classes; namely coordination complexes and organometallic derivatives ( Fig. 1). The first class comprises mononuclear hydroxo complexes $[Au(bipy^{R})(OH)_2][PF_6]$ ($bipy^{R}$ = 2,2′-bipyridine, **1**; 4, 4′-di-*tert*-butyl-2,2′-bipyridine, **2**) and dinuclear oxo-bridged complexes $[(bipy^{R})_2Au_2(\mu\text{-}O)_2][PF_6]_2$ ($bipy^{R}$ = 2,2′-bipyridine, **3**; 4,4′-di-*tert*-butyl-2,2′-bipyridine, **4**), all featuring chelating 2,2′-bipyrines as auxiliary ligands and anionic O-donor groups.[24] The hydroxo complexes **1** and **2** are obtained from reaction of the dichloro complexes $[Au(bipy^{R})Cl_2][PF_6]$ with $Ag_2O$ in water solution. Compounds **3** and **4** are obtained from **1** and **2**, respectively, by condensation reaction in anhydrous solvents. Vice versa, **1** and **2** can be regenerated from **3** and **4** in water solution at reflux. The second class includes two C(4)-metallated derivatives of 1-phenyl-pyrazole ($pz^{Ph}$), namely *cis*-$[Au(pz^{Ph}\text{-}H)(PPh_3)Cl_2]$ (**5**) and *trans*-$[Au(pz^{Ph}\text{-}H)(py^{2Me})Cl_2]$ (**6**) ($py^{2Me}$ = 3,5-dimethylpyridine), and $[Au(py^{dmb}\text{-}H)(OAc)_2]$ (**7**), a cyclometallated derivative of 2-(1,1-dimethylbenzyl)-pyridine ($py^{dmb}$). Complexes **5** and **6** are obtained by reaction of the common polymeric precursor $[Au(pz^{Ph}\text{-}H)Cl_2]_n$ with $PPh_3$ and $py^{2Me}$, respectively,[25] while complex **7** is obtained by reaction of the corresponding dichloro complex $[Au(py^{dmb}\text{-}H)Cl_2]$[26] with two equivalents of AgOAc.

### 3.2. Biological evaluation

Gold(III) is thermodynamically less stable than gold(I) or gold(0) and as a result, many gold(III) complexes are readily reduced, thereby acting as strong oxidising agents in biological enviroments.[1] The reduction of gold(III), driven by biologically occurring reductants such as thiols, has been well described and is typically associated with toxicity in cell-based assays.[1] Although not as extensively studied as gold(I) complexes, gold(III) complexes have been evaluated as potential inhibitors of several diseases including HIV-1.[14–21] As such, it was of interest to delineate the effect of gold(III) complexes, and in particular the influence of gold(III) reduction, within direct assays typically used to identify modulators of HIV-1 enzyme activity.

The gold(III) tetrachlorides $HAuCl_4$ and $KAuCl_4$ were selected for this study as they are widely used precursors for the synthesis of gold(III) complexes. Additionally, $KAuCl_4$ has been previously shown to inhibit the activity of HIV-1 RT in the direct RT assay.[20] In that study, the authors found the gold(III) precursor to inhibit RT activity by 48% at a concentration of 6 μM but noted that this inhibition was significantly less than the inhibition of their test compounds. The authors of that study did not, however, attribute the inhibition/reduction of RT activity to protein oxidation. Within our laboratory, both $HAuCl_4$ and $KAuCl_4$ reduced HIV-1 RT activity, as measured in the direct RT assay. At an initial single-dose concentration of 10 μM, $HAuCl_4$ reduced RT activity by ~100% (Table

1). Through further dose-dependent studies, a sub-micromolar $IC_{50}$ (0.947 μM) was obtained for this gold salt (Table 1). Similarly, $KAuCl_4$ was also effective in reducing the activity of the recombinant RT enzyme. At a single-dose concentration of 10 μM, the gold(III) tetrachloride reduced RT activity by 99.79% while a sub-micromolar $IC_{50}$ of 0.983 μM was determined (Table 1). In our hands, $KAuCl_4$ reduced RT activity by >80% at the 6 μM single-dose evaluation (data not shown).

The gold(III) tetrachlorides reduced HIV-1 RT activity but gold(III) complexes with protecting ligands are more likely to be evaluated and screened for activity within biological assays. As such, a small number of gold(III) complexes (Fig. 1) were tested for effect within the direct HIV-1 RT assay. Notably, each of the 7 gold(III) complexes affected the assay outcome, in each case reducing the RT activity detected by >90% at a single dose concentration of 10 μM. As observed with the gold(III) tetrachlorides, reduction in RT activity was dose-dependent and $IC_{50}$'s ranging from 0.461 to 8.796 μM were obtained (Table 1). The influence on RT activity was different for each compound, thus illustrating that the reduction of the gold(III) was dependent on the coordinated ligand. Indeed, as anticipated by previous electrochemical studies on gold(III) adducts[36] and cyclometalated derivatives,[37] the less oxidant species, i.e. the cyclometalated derivative **7**, is the least active. Nonetheless, reduction of gold(III) was sufficiently rapid in all samples to affect the functioning of the recombinant RT enzyme within the 60 min period that the enzyme was exposed to each complex. Despite the sub-micromolar inhibition of HIV-1 RT activity within the direct assay, none of the gold complexes yielded notable cellular antiviral activity (Table 2). In cell-based assays, anti-viral dose curves were found to closely parallel the cytotoxicity dose response curves. The highest SI value achieved was 5.95 and therefore none of the gold(III) complexes tested were considered selective inhibitors of viral replication (Table 2).

In comparison with the reduction in direct RT activity observed with gold(III) complexes, no appreciable effect was observed with either the gold(I) phosphine complex $Au(dppe)_2Cl$ or with the commercial gold(I) drug Auranofin at a single dose concentration of 10 μM (Table 1). Additionally, 30 gold(I) complexes were also evaluated within the direct RT assay at a single dose concentration of 10 μM. None of these complexes exceeded the 50% inhibition cut-off value, with a highest inhibition of only 22.68% obtained (data not shown). These findings suggest that the effect noted is due to the oxidation state of gold(III) and not due to the presence of gold or the binding of gold to the enzyme.

While the HIV-1 RT enzyme is considered to be non-sensitive to oxidation, the data discussed above suggests that gold(III) reduces enzymatic activity within the direct RT assay through non-specific oxidation of the enzyme. The exact target of the oxidation remains to be elucidated but the cysteine thiols represent likely targets. In a previously published study, Persichini and co-workers attributed dose-dependent inhibition of HIV-1 RT by nitric oxide (NO) donors at concentrations of 0.01–1 mM to the oxidation of the two Cys residues (C38 and C280) present on the p66 subunit of HIV-1 RT.[38] Specifically, it was suggested that the NO-mediated modification of HIV-1 RT proceeded through oxidation of the free thiols beyond sulfenic, sulfinic or sulfonic acid.[38] This hypothesis appears to be corroborated in our study as the simultaneous addition of DTT (10 mM) to the reaction mixture containing the RT enzyme neutralised the inhibitory effect of $HAuCl_4$ and $KAuCl_4$ in the direct RT assay while the inhibitory activity of AZT-tp and nevirapine was not altered (Table 1). It is noted, however, that several detailed studies on the functionality of the two Cys residues have demonstrated that these residues are not critical to RT activity and that modification would not alter the DNA polymerase activity of the enzyme.[39,40] Alternatively, RT function may therefore be altered by gold(III) through oxidation of the methionine (Met) residues. As shown in Table 1, the M184V RT mutant retains <25% more activity than the wild-type RT enzyme and the $p66^{E>Q}$/p51 mutant RT when treated with gold(III) complexes. As the methionine at position 184 is only one of six p66 subunit methionines (Fig. 2a), the reduced RT function may be due, at least in part, to the oxidation of Met residues.

The reduction of inhibitory activity observed in the presence of 10 mM DTT may result from DTT protection of enzymatic sulphurs or from a direct reaction between DTT and the metal complex, preventing any further interaction between the gold(III) compound and the protein. The latter possibility has been previously postulated[23,41] and the effect of DTT on gold(III) complex **1** was investigated further in this study. Here, successive equivalent amounts of reducing agent DTT were directly added to a solution of gold(III) complex and monitored via NMR. Although changes on the metal centre could not be observed directly, it was possible to monitor the signals associated with the coordinated ligand. In this case, the addition of DTT to complex **1** induced an upfield shift of the aromatic signals, inferring interaction between DTT and the gold (III) complex (Fig. 3). The upfield shift observed corresponded to changes in the chemical surroundings of the protons involved, especially those of position 6 (and 6′) in the bipyridyl ligand, affected by the lone pair electron density availability of the neighbouring N atoms. These shifts are in agreement with those observed during incubation of the complexes at 37 °C over 72 h (with particularly faster changes observed in the presence of serum

**Table 1**
The direct inhibition of HIV-1 reverse transcriptase and HIV-1 integrase enzymatic activity by the gold precursors and gold(III) complexes evaluated in this study

| Sample | Recombinant wild-type RT | | $p66^{E>Q}$/p51 Mutant RT | M184V Mutant RT | Recombinant HIV-1 IN |
|---|---|---|---|---|---|
| | % Inhibition at 10 μM (+10 mM DTT) | $IC_{50}$[a] ± SE[b] (μM) | % Inhibition (10 μM) | % Inhibition (10 μM) | % Inhibition at 10 μM (+10 mM DTT) |
| AZT-tp | 98.346 ± 1.870 (100.004 ± 0.223) | — | — | — | — |
| Nevirapine | 99.676 ± 0.240 (98.652 ± 3.969) | — | — | — | — |
| $HAuCl_4$ | 100.32 ± 0.27 (28.069 ± 2.151) | 0.947 ± 0.209 | — | — | 80.551 ± 5.049 (12.745 ± 0.984) |
| $KAuCl_4$ | 99.79 ± 0.76 (26.379 ± 1.981) | 0.983 ± 0.185 | — | — | 82.582 ± 2.151 (5.176 ± 1.489) |
| Audppe | 20.64 ± 2.27 | — | — | — | — |
| Auranofin | 26.362 ± 0.141 | — | — | — | — |
| **1** | 97.455 ± 1.572 | 0.502 ± 0.018 | 99.295 ± 0.834 | 75.826 ± 2.014 | — |
| **2** | 97.659 ± 0.872 | 0.461 ± 0.053 | 97.230 ± 1.708 | 67.500 ± 1.699 | — |
| **3** | 96.368 ± 2.792 | 0.503 ± 0.042 | 97.183 ± 4.992 | 75.242 ± 6.700 | — |
| **4** | 98.618 ± 1.313 | 0.660 ± 0.000 | 97.124 ± 2.354 | 65.351 ± 1.932 | — |
| **5** | 99.159 ± 1.171 | 1.381 ± 0.220 | — | — | — |
| **6** | 97.260 ± 1.077 | 1.054 ± 0.001 | — | — | — |
| **7** | 95.263 ± 3.414 | 8.796 ± 0.017 | — | — | — |

[a] $IC_{50}$, 50% inhibitory concentration. Calculated as the concentration of compound required to reduce the activity of reverse transcriptase (RT) enzyme by 50%.
[b] SE, Standard error. Standard error of the mean for at least three separate experiments.

**Table 2**
The cytotoxicity data ($CC_{50}$), anti-HIV activity ($EC_{50}$) and selectivity index (SI) of the gold(III) complexes in PBMC's.

| Complex | [a]$CC_{50}$ in PBMCs (μM) | [b]$EC_{50}$ versus HIV-1 Ba-L (μM) | $EC_{50}$ versus HIV-1 NL4-3 (μM) | [c]SI value |
|---|---|---|---|---|
| **1** | 40.6 | 14.1 | 37.2 | 2.87<br>1.09 |
| **2** | 6.28 | 3.66 | 4.43 | 1.71<br>1.42 |
| **3** | 18.7 | 8.22 | 13.0 | 2.27<br>1.43 |
| **4** | 2.94 | 1.83 | 3.15 | 1.61<br><1.00 |
| **5** | 2.45 | 0.41 | 1.12 | 5.95<br>2.19 |
| **6** | 56.5 | 23.5 | 40.8 | 2.41<br>1.38 |
| **7** | 8.30 | 1.80 | 7.86 | 4.60<br>1.06 |

[a] $CC_{50}$, 50% cytotoxic concentration. Calculated as the concentration of compound required to reduce cell viability by 50%.
[b] $EC_{50}$, 50% effective concentration. Defined as the concentration of compound required to reduce HIV-1 replication by 50%.
[c] SI, selectivity index. Calculated as the ratio of $CC_{50}/EC_{50}$.

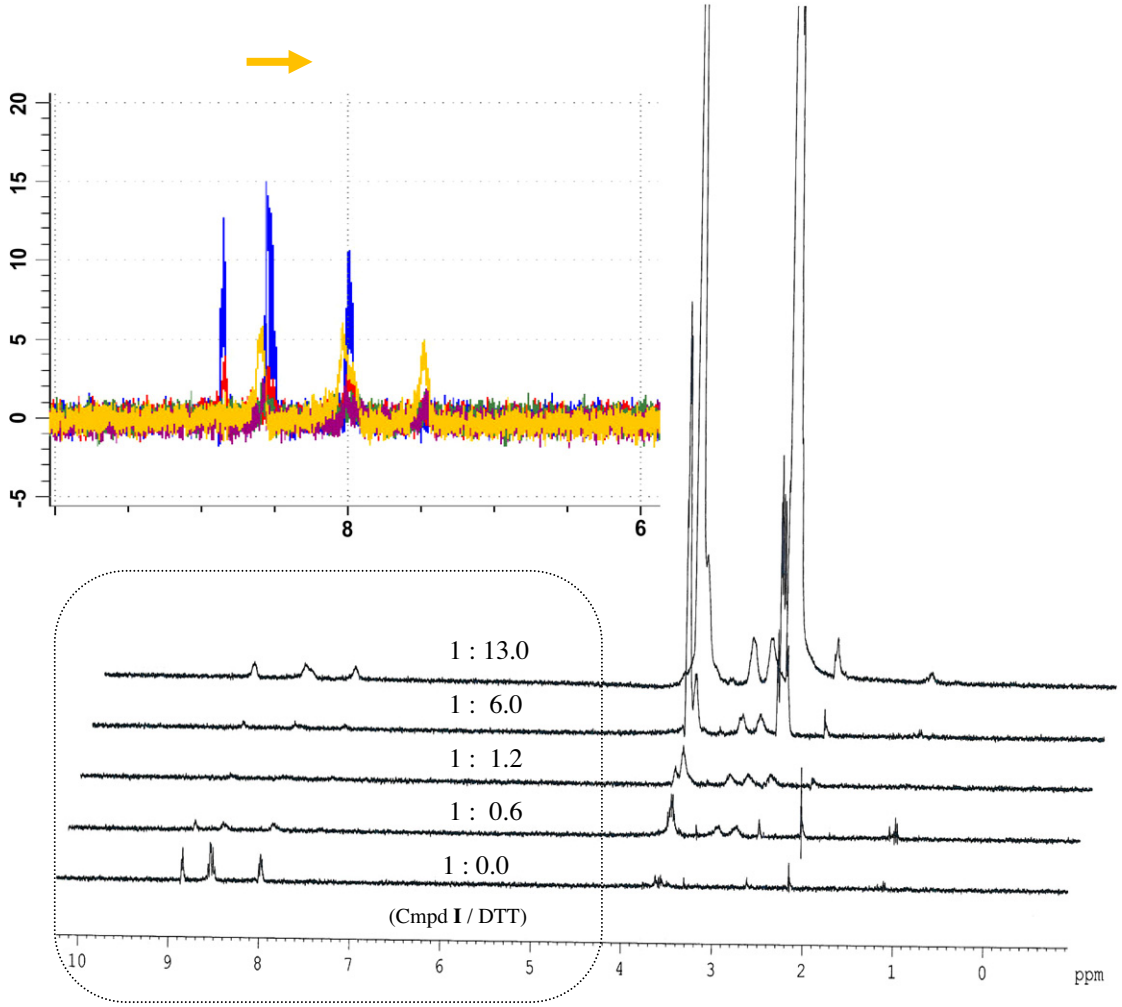


**Figure 3.** Addition of molar equivalents of DTT to test compound **1** (2.89 mM) in deuterated PBS. Signals associated with **1** are highlighted (other signals correspond to DTT, DMSO and PBS solution added). Overlapped spectra are shown in colour above (blue to orange: 0- to 13-fold molar excess DTT).

proteins) and those of the decomposed complex (results not shown).

Further addition of DTT (6:1) resulted in cloudiness of the NMR solution, while a 13-fold excess induced the appearance of colloidal gold, indicating that complex **1** had been reduced, at least in part. These observations are also in agreement with those reported for a structurally-related gold(III) compound, which afforded an adduct between two gold(I) atoms, a tetrapetide and DTT when treated with fivefold excess of the reducing agent, suggesting reduction of the gold centre and release of the ligand.[42] Under

the experimental conditions tested, (10 mM; 100-fold excess) it is assumed that the gold(III) has fully reacted with DTT and therefore can no longer influence the protein activity. As further evidence that DTT interacts with the gold compounds rather than reversing the gold-mediated oxidation, we found that the addition of excess DTT post incubation with the gold(III) compounds did not restore enzyme activity (data not shown). Similarly, Persichini and co-workers observed that the addition of DTT to RT inactivated by NO donors did not repair enzymatic activity.[38]

The gold(III) tetrachlorides evaluated in this study are strong oxidisers of HIV-1 RT, more so than the NO donors described by Persichini and co-workers,[38] and may be non-selective. In a direct HIV-1 integrase ELISA assay, the gold(III) tetrachlorides had significant inhibitory effect within the assay, reducing activity of the enzyme by >80% at the single-dose concentration of 10 μM (Table 1). As depicted in Figure 2b, the full length HIV-1 integrase has five cysteine residues and four methionine residues, of which three cysteines and two methionines are in the central catalytic domain (CCD). As such, reduction of enzymatic activity may occur through oxidation of protein residues similarly described for HIV-1 reverse transcriptase but further investigation needs to be completed.

## 4. Conclusion

In this study, gold(III) complexes were identified as strong oxidisers of the HIV-1 RT, an enzyme which is generally not sensitive to oxidation due to the fact that it has only two cysteinyl residues neither of which are critical to RT function. This strong oxidation potential should also be taken into consideration when testing gold(III) complexes in direct assays aimed at identifying HIV-1 enzyme inhibitors. The addition of DTT to the reaction mixture may facilitate the distinction between true gold(III) inhibitors of RT activity and non-specific oxidising agents. Notably, HIV-1 integrase activity was also affected by the gold(III) compounds evaluated in this study, suggesting that modification of the direct integrase assay (with DTT or other) may also be necessary to eliminate false positives arising from the reduction of the gold(III) metal centre to a lower oxidation state.

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