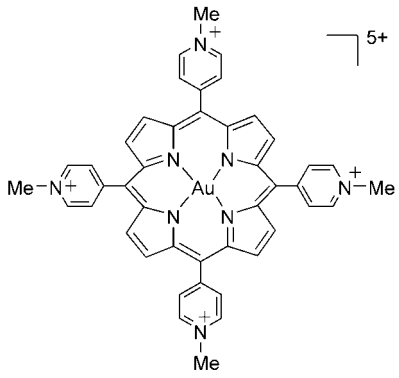
In Vitro Inhibition of Human Immunodeficiency Virus Type-1 (HIV-1) Reverse Transcriptase by Gold(III) Porphyrins

Raymond Wai-Yin Sun, Wing-Yiu Yu, Hongzhe Sun, and Chi-Ming Che*[a]

The medicinal uses of gold compounds have been a subject of extensive studies.[1] Notable examples include auranofin, which is a Au^{I}-thiolate complex, for clinical treatment of rheumatoid arthritis. Although gold(III) compounds have long been anticipated as promising candidates for drug development, limited success has been achieved due to their poor solution stability under physiological conditions.[2] Previously we showed that porphyrin is a good auxiliary ligand for stabilizing gold(III), and gold(III) porphyrins exhibit remarkable solution stability under physiological conditions and potent anticancer activities against a panel of human cancers in vitro.[3]

In 1996, Shapiro and Masci reported that an HIV patient receiving auranofin for psoriatic arthritis treatment showed an elevated $CD4^+$ T-cell count, and anti-HIV activity of gold compounds was implicated.[4] Stimulated by this finding, we explored the inhibition of HIV-1 reverse transcriptase with some structurally defined gold(III) compounds. Herein is described that $[Au^{III}(TMPyP)]Cl_5$ (**1a**, $(H_2TMPyP)^{4+}$ = *meso*-tetrakis(*N*-methylpyridinium-4-yl)porphyrin) is a potent inhibitor of HIV-1 reverse transcriptase in vitro with an IC_{50} value = 0.31 μM. In combination with 3′-azido-3′-deoxythymidine triphosphate (AZT-TP), **1a** can effect significant HIV-1 reverse transcriptase (RT) inhibition at nanomolar concentration. Prior to this work,



$[Au^{III}(TMPyP)]^{5+}$ (**1a**)

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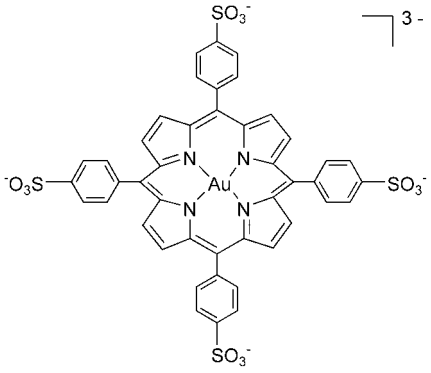
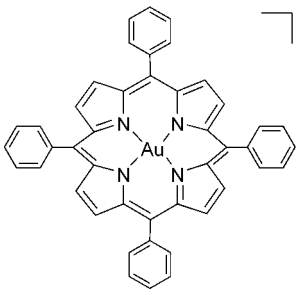
Paterson and Kappas reported that heme and some synthetic metalloporphyrins are inhibitors of HIV-1 reverse transcriptase in vitro.[5]

Previously we showed that gold(III) porphyrins such as [Au^{III}(TPP)]Cl (**1c**) are stable in physiological buffer medium,[3]



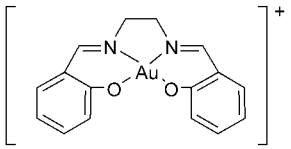
[Au^{III}(TPPS)]$^{3-}$ (**1b**)



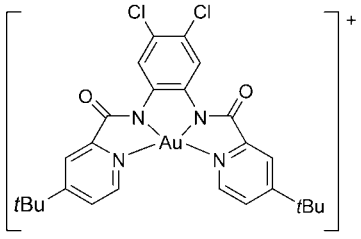
[Au^{III}(TPP)]$^{+}$ (**1c**)

and no significant decomposition was observed in the presence of excess glutathione (GSH). In this work, [Au^{III}(TMPyP)]Cl$_5$ (**1a**) and Na$_4$[Au^{III}(TPPS)]Cl (**1b**, (H$_2$TPPS)$^{4-}$ = *meso*-tetrakis(4-sulfonatophenyl)porphyrin) were found to be stable in Tris buffer saline (TBS; pH 7.2) at room temperature, based on UV/Vis spectroscopic studies. No significant UV/Vis and ^{1}H NMR spectral changes were observed when treating **1a**/**1b** with excess GSH.

Analogous to porphyrins, Schiff bases[6] and bis(pyridyl)carboxamides[7] are also strong chelating σ-donor ligands. In this work, we also prepared gold(III) complexes containing *N,N′*-ethylenebis(salicylideneimine) (H$_2$salen) and 4,5-dichloro-1,2-bis(2-(4-*tert*-butylpyridine)carboxamido)benzene (H$_2$dcbpb) as auxiliary ligands. The [Au^{III}(salen)]Cl (**2**) and [Au^{III}(dcbpb)]Cl (**3**)



[Au^{III}(salen)]$^{+}$ (**2**)



[Au^{III}(dcbpb)]$^{+}$ (**3**)

complexes were prepared by treating K[Au^{III}Cl$_4$] with the corresponding ligands in refluxing acetonitrile and acetic acid, respectively.[8] The complexes were characterized by ^{1}H NMR spectroscopy, FAB-MS (for **2**: *m/z* = 463 [*M*$^+$]; for **3**: *m/z* = 694 [*M*$^+$]) and elemental analyses.

Complexes **2** and **3** are stable in TBS/MeCN (9:1; pH 7.2) and no significant spectral changes were evident upon standing the solutions at room temperature for 4 h (see Supporting Information). However, adding GSH (2 mM) to the solutions of **2** and **3** in TBS resulted in spontaneous spectral changes (Figure 1), accompanied by formation of colloidal gold and some light yellow precipitates. FAB-MS analysis of the precipitates revealed the molecular ions of the free H$_2$salen (*m/z* = 267) for **2** and H$_2$dcbpb (*m/z* = 498) for **3**. These findings indicate that **2** and **3** underwent extensive demetallation and reduction of gold(III) to colloidal gold upon treatment with GSH.

In this study, the activities of the gold(III) complexes toward HIV-1 reverse transcriptase inhibition were measured by using an ELISA method developed by Eberle and Seibl.[9] The method was performed by incubating the enzyme with digoxigenin-labeled deoxyuridine-5-triphosphate (dUTP) and the biotin-labeled analogue during reverse transcription starting from the template/primer hybrid poly(A)·oligo(dT)$_{15}$. After reverse transcription, the newly synthesized DNA was immobilized into streptavidin-coated ELISA wells, and the incorporation of the dioxigenin-labeled dUTP was assayed by a chemiluminescence method.

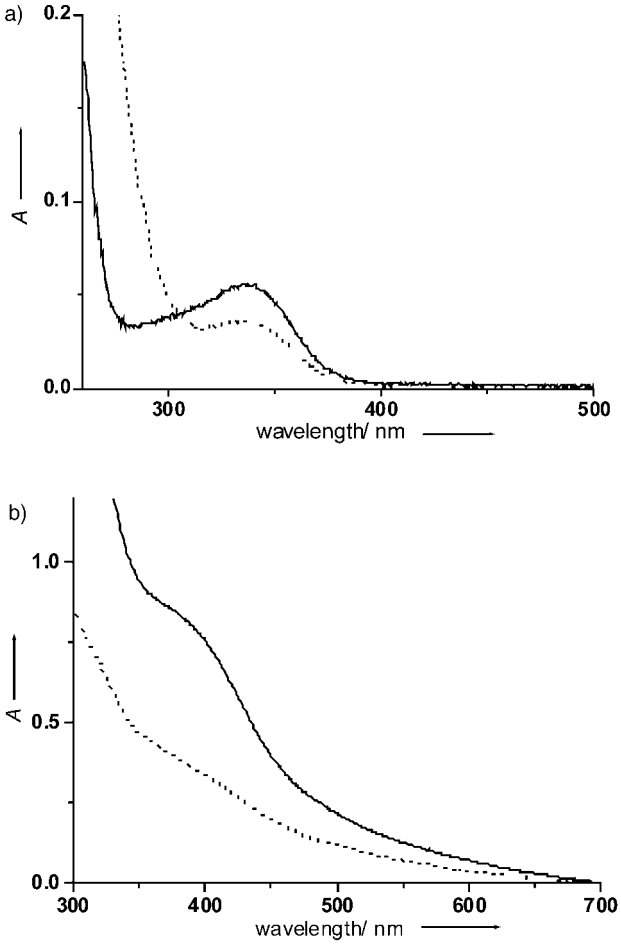


Figure 1. *UV-visible spectra of a)* ***2*** *(10 μM) and b)* ***3*** *(10 μM) in 2 mM GSH TBS/MeCN (19:1) at* t = *0 h (solid line) and* t = *48 h (dotted line).*

Upon treatment of HIV-1 RT in lysis buffer (2 ng, 128.7 μL) with $[Au^{III}(TMPyP)]Cl_5$ (**1a**; 50 μM) dissolved in TBS at 37 °C, significant RT inhibition (~95%) was observed after 30 min incubation compared with the drug-free control (i.e., 0% inhibition). The HIV-1 RT inhibitory activity of **1a** was found to be dose-dependent: 95% inhibition at 50 μM, 76% at 10 μM, 67% at 5 μM, 58% at 1 μM, 53% at 0.5 μM, and 38% at 0.1 μM. The IC_{50} value was evaluated to be 0.31 ± 0.05 μM (Table 1, entry 1). Likewise, $Na_4[Au(TPPS)]Cl$ (**1b**) also exhibited effective HIV-1 RT inhibition with an IC_{50} value (0.57 ± 0.09 μM) comparable to that of **1a** (entry 2). It should be noted that the free-base porphyrins (i.e., $(H_2TMPyP)^{4+}$ and $(H_2TPPS)^{4-}$) display substantially lower HIV-1 RT inhibitory activities (see below). In this work, the HIV-1 RT inhibitions by **1c**, **2** and **3** were also examined, and the corresponding IC_{50} values were found to be 28.4 ± 1.8 μM (**1c**), 0.33 ± 0.03 μM (**2**) and 0.47 ± 0.09 μM (**3**; entries 3–5).

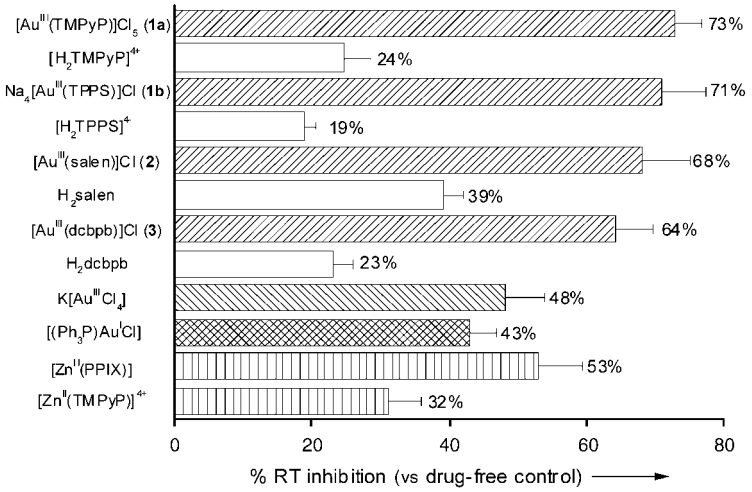


Figure 2. *Comparative study of HIV-1 RT inhibition by various related compounds with concentration fixed at 6 μM. For comparison, the inhibitory effects of $KAu^{III}Cl_4$, $[(Ph_3P)Au^{I}Cl]$ and some zinc(II) porphyrins are also shown.*

Table 1. *Inhibitory concentration (IC_{50}) values of various gold complexes against HIV-1 RT.*

	compound	$IC_{50} \pm SE$ [μM^{-1}][a]
1	**1a**	0.31 ± 0.05
2	**1b**	0.57 ± 0.09
3	**1c**	28.4 ± 1.8
4	**2**	0.33 ± 0.03
5	**3**	0.47 ± 0.09
6	$[Zn^{II}(PPIX)]$	4.98 ± 0.93
7	AZT-TP	0.069 ± 0.01

[a] Expressed as mean ± SE of at least three determinations. HIV-1 RT = HIV-1 reverse transcriptase.

Paterson and co-workers reported that $[Zn^{II}(PPIX)]$ (H_2PPIX = protoporphyrin IX) inhibited HIV-1 RT activity in vitro.[5a] In this work, we have also determined the corresponding IC_{50} value (4.98 ± 0.93 μM) using the ELISA assay (Table 1, entry 6). As shown in Table 1, $[Zn^{II}(PPIX)]$ is at least tenfold less effective than the gold(III) porphyrins in HIV-1 RT inhibition.

By employing a fixed drug concentration (6 μM), the HIV-1 RT inhibitory activities of various related compounds were compared, and the results are depicted in Figure 2. Around 70% HIV-1 RT inhibition was observed for **1a**, **b** and **2**, whereas **3** was found to effect 64% RT inhibition under identical conditions. Apparently, the presence of a gold atom is critical for the observed HIV-1 RT inhibition, since all the free-base ligands are largely inactive (<30% inhibition). For comparison, $KAu^{III}Cl_4$ was found to achieve only 48% inhibition of the HIV-1 RT activities, compared with 64–73% inhibition attained by **1**–**3**. We found that **1**–**3** are more effective in vitro HIV-RT inhibitors than $[(Ph_3P)Au^{I}Cl]$,[10] which produced only 43% inhibition at 6 μM. As noted earlier, $[Zn^{II}(PPIX)]$ is a less effective HIV-1 RT inhibitor than the gold(III) complexes (c.f. 53% RT inhibition by $[Zn^{II}(PPIX)]$ to 70% RT inhibition by **1**–**3** under identical conditions). Indeed, the unique activity of gold(III) is further exemplified by the fact that $[Zn^{II}(TMPyP)]Cl_4$ was found to produce only 32% RT inhibition (c.f. 70% for **1a**) at 6 μM.

Acute cytotoxicities of the gold(III) complexes to human T-cells, which are the host cells for HIV replication, have been evaluated. The percentages of cell survival at various doses of the gold complexes were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay,[11] and the cytotoxicity profiles are shown in Figure 3. Importantly, **1a** and **1b** did not exert significant acute cytotoxicities to human T-cells, with >90% cell survival being registered at drug concentration up to 96 μM, which corresponds to 16-fold of their IC_{70} values (ca. 6 μM) for HIV-1 RT inhibition. In contrast, >90% cell death was found when treating the T-cells with **2** and **3** at 96 μM level. At about 3 μM, $[Au^{III}(TPP)]Cl$ (**1c**) showed severe cytotoxicity to the T-cells, with 80% cell death being observed (c.f. $IC_{50} = 28$ μM for HIV-1 RT inhibition). With human lung fibroblasts (CCD-19Lu) as model normal cells, **1a** and **1b** were found to be nontoxic (i.e., >90% cell survival at

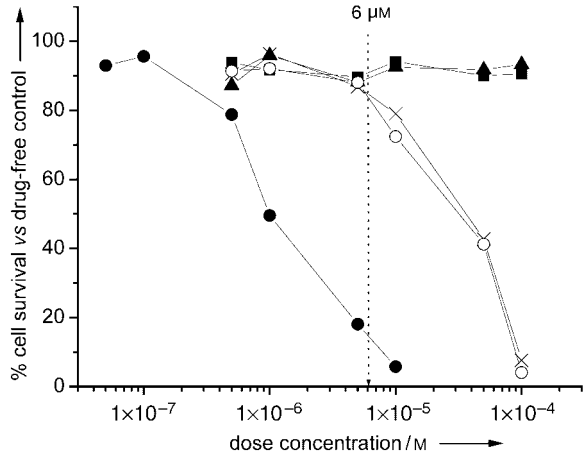


Figure 3. *Cytotoxicity profiles of complexes **1**–**3** toward human T-cells. A plot of % cell survival versus log [concentration of gold(III) complex]; ■ = **1a**, ▲ = **1b**, ● = **1c**, ○ = **2**, × = **3**.*

concentrations $>96\ \mu M$) to healthy cells (see Supporting Information).

Complex **1a** was chosen for further examination because of its favourable solution stability and cytotoxicity profile. As shown in Figure 4, only 13.8–38.8% enzyme inhibition was

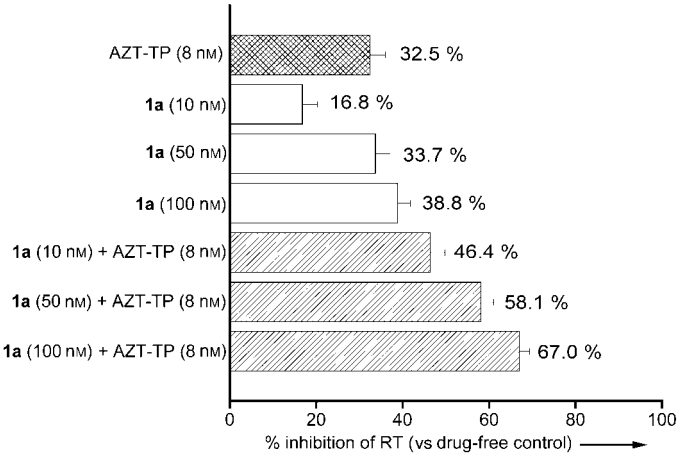


Figure 4. *HIV-1 RT inhibitory activities of $[Au^{III}(TMPyP)]Cl_5$ (**1a**) alone and in combination with AZT-TP.*

produced when treating HIV-1 RT with **1a** (10, 50 and 100 nM) or AZT-TP (8 nM) alone. Strikingly, when **1a** was used in combination with AZT-TP, a significant additive effect on HIV-1 RT inhibition was observed. Up to 67% enzyme inhibition was achieved by employing the "**1a** (100 nM) + AZT-TP (8 nM)" combination. With reference to the IC_{70} value (6 μM) produced by **1a** alone, the "**1a** + AZT-TP" protocol has lowered the dosage requirement of the gold(III) porphyrin by 60-fold in attaining comparable enzyme inhibition.

To account for the synergistic HIV-1 RT inhibition by **1a** and AZT-TP, it seems plausible that the gold(III) porphyrin binds to certain sites near the active site of the enzyme. Paterson and co-workers previously proposed that the $[Zn^{II}(PPIX)]$-mediated HIV-1 RT inhibition would be associated with binding to the connection domain sequence 398–407 (WETWWTEYWQ).[5a] In this work, UV/Vis absorption titration studies revealed that **1a** binds strongly to the WETWWTEYWQ sequence in aqueous buffer medium. The sequence was obtained from a commercial source (Biopeptide Co, USA); HPLC purified and characterized by mass spectrometry ($m/z = 1515$ $[M^+]$) prior to uses. Addition of the peptide (0–5 μM) to **1a** produced isosbestic spectral changes (isosbestic points at 314, 417, 508 and 532 nm) with significant hypsochromicity (58%) and small bathochromic shift (7 nm) of the Soret band (see Supporting Information). By using the absorbance data of the Soret band, the linear plot ($R = 0.99$) of [peptide]/$\Delta\varepsilon_{ap}$ versus [peptide] gave the binding constant $K_b = (7.9 \pm 0.7) \times 10^4\ dm^3\,mol^{-1}$ at 292 K.[12] A comparable K_b value ($(1.3 \pm 0.1) \times 10^5\ dm^3\,mol^{-1}$ at 292 K) was obtained for the $[Zn^{II}(PPIX)]$–WETWWTEYWQ interaction, based on a UV/Vis absorption titration study. Interestingly, no significant binding of **1b** with the peptide was observed according to UV/Vis spectral titration. Given that **1b** is an effective HIV-1 RT inhibitor, the poor binding affinity to WETWWTEYWQ suggests an alternative binding site for the complex.

While **1a** was found to bind strongly to the WETWWTEYWQ peptide, its HIV-1 RT inhibitory activity was unaffected by tenfold excess of the peptide (see Supporting Information). In contrast, significant reduction of HIV-1 RT inhibitory activity of $[Zn^{II}(PPIX)]$ was observed in the presence of excess WETWWTEYWQ peptide. This result suggests that **1a** and $[Zn^{II}(PPIX)]$ are unlikely to have the same binding site (i.e., the connection domain sequence 398–407) for HIV-1 RT inhibition.

In conclusion, $[Au^{III}(TMPyP)]Cl_5$ (**1a**) is an effective inhibitor of HIV-1 reverse transcriptase in vitro with effective inhibitory concentration at the submicromolar level. When using human T-cells and fibroblasts as a model for healthy cells, no significant cytotoxicity of **1a** was observed.

Experimental Section

Materials: Analytical-grade organic solvents and double-distilled deionized water were used throughout the experiments. *meso*-Tetrakis(*N*-methyl-4-pyridyl)porphyrin ($(H_2TMPyP)^{4+}$) tetratosylate salt and *meso*-tetrakis(4-sulfonatophenyl)porphyrin ($(H_2TPPS)^{4-}$) tetrasodium salt were obtained commercially and used as received. *meso*-Tetraphenylporphyrin (H_2TPP) was synthesized and purified by literature methods.[13] *N,N'*-Ethylenebis(salicylideneimine) (H_2salen)[14] and 4,5-dichloro-1,2-bis(2-(4-*tert*-butylpyridine)carboxamido)benzene (H_2dcbpb)[15] were prepared by the reported methods. The preparation of $[Au^{III}(TPP)]Cl$ (**1c**) has been reported elsewhere.[3]

Isolated T-cells were prepared from the buffy coat obtained from the Hong Kong Red Cross Blood Transfusion Service, with permission for their use for research purposes from the blood donor. Each unit of the buffy coat prepared from whole blood (450 mL) contained approximately 5×10^8 cells. To isolate the T-cells, the buffy coat (15 mL) was transferred to a 50 mL Eppendorf tube under sterile condition and was diluted by serum-free RPMI 1640 culture medium (1:1, *v/v*) ratio. Ficoll® solution (15 mL) was then gently added to the diluted blood, and the mixture was separated in a centrifuge at 800 *g* for 25 min at room temperature. The peripheral blood mononuclear cells (PBMCs) at the interface were washed five times with cold RPMI culture medium. The washed PBMCs were first spun at 500 *g* for 7 min, then at 250 *g* for 7 min and finally at 200 *g* to obtain a cell pellet. The PBMCs were resuspended in ammonium chloride lysis buffer (ACK buffer) and incubated at room temperature for 5 min to lyse the remaining red blood cells. A human-T-cell enrichment column (R&D Systems, Minneapolis, MN) was then used according to the manufacturer's instructions to rapidly purify the T-cells by using high-affinity negative selection.

The peptide (WETWWTEYWQ) was purchased from Biopeptide Co. (San Diego, CA) and was HPLC-purified before use. The normal human-lung fibroblast cell line (CCD-19 Lu) was obtained commercially from American Type Culture Collection (ATCC). Cell culture flasks and 96-well microtitre plates were purchased from Nalge Nunc International. Culture medium, other medium constituents and phosphate buffered saline (PBS) were obtained from Gibco BRL. Cell Proliferation Kit I (MTT) was purchased from Roche.

Instrumentation: Absorption spectra were recorded on a Perkin–Elmer Lambda 19 or a Varian Cary 50 UV-visible spectrophotometer equipped with a PCB-150 water circulator. ^{1}H NMR spectra were recorded on Bruker DPX-300 or DPX-500 NMR spectrometers. Positive ion FAB mass spectra were recorded on a Finnigan MAT95 mass spectrometer by using 3-nitrobenzyl alcohol as matrix. Electrospray ionization (ESI) mass spectrometry was performed on a Finnigan LCQ quadrupole ion-trap mass spectrometer. Elemental analyses were performed by the Institute of Chemistry of the Chinese Academy of Sciences.

Preparation of gold(III) complexes: $[Au^{III}(TMPyP)]Cl_5$ (**1a**)[15] and $Na_4[Au^{III}(TPPS)]Cl$ (**1b**)[16] were prepared according to the literature.

For **1a**: Yield = 34%; ^{1}H NMR 300 MHz (D_2O): $\delta = 9.52$ (s, 8H), 9.33 (d, $J = 8.0$ Hz, 8H), 9.03 (d, $J = 6.4$ Hz, 8H) 2.07 (s, 12H); UV/Vis (TBS): λ_{max} ($\log\varepsilon$) = 405 (4.89), 522 (3.98), 556 nm^{-1} (3.64); FAB-MS: $m/z = 1017$ [$M^+ - Cl$]; elemental analysis calcd (%) for $C_{44}H_{60}N_8O_{12}Cl_5Au$: C 41.70, H 4.77, N 8.84; found: C 41.98, H 4.65, N 8.57.

For **1b**: Yield = 42%; ^{1}H NMR 300 MHz (D_2O): $\delta = 8.62$ (s, 8H), 7.69 (d, $J = 7.8$ Hz, 8H), 8.24 (d, $J = 8.6$ Hz, 8H); UV/Vis (TBS): λ_{max} ($\log\varepsilon$): 407 (5.34), 521 nm^{-1} (4.39); FAB-MS: $m/z = 1217$ [$M^+ + 4Na - Cl$]; elemental analysis calcd (%) for $C_{44}H_{40}N_4O_{20}ClS_4Na_4Au$: C 37.82, H 2.89, N 4.01; found: C 37.53, H 3.01, N 4.07.

$[Au^{III}(salen)]Cl$ (**2**) and $[Au^{III}(dcbpb)]Cl$ (**3**) were synthesized according to the reported procedures.[8]

For **2**: Yield = 23%; ^{1}H NMR 300 MHz ([D_6]DMSO): $\delta = 4.19$ (s, 4H), 6.98–7.80 (m, 8H), 8.95 (s, 2H); UV/Vis (DMSO): λ_{max} ($\log\varepsilon$) = 347 nm^{-1} (3.95); FAB-MS $m/z = 498$ [$M^+ - Cl$]; elemental analysis calcd (%) for $C_{16}H_{14}N_2O_2ClAu$: C 38.55, H 2.83, N 5.62; found: C 38.39, H 2.81, N 5.58.

For **3**: Yield = 23%; UV/Vis (DMSO) λ_{max} ($\log\varepsilon$) = 398 nm^{-1} (4.09); ^{1}H NMR 300 MHz ([D_6]DMSO): $\delta = 1.62$ (s, 18H), 6.48–8.15 (m, 8H); FAB-MS $m/z = 729$ [$M^+ - Cl$]; elemental analysis calcd (%) for $C_{26}H_{26}N_4O_2Cl_3Au$: C 42.85, H 3.60, N 7.69; found: C 42.63, H 3.56, N 7.67.

Evaluation cytotoxicity of the gold(III) complexes to human T-cells: Human T-cells were maintained in a RPMI 1640 medium that was supplemented with 2 mM L-glutamine and 10% foetal bovine serum. The whole cultures were kept at 37 °C in a 5% CO_2/95% air humidified atmosphere. The supplemented culture medium (90 μL) with cells (3×10^5 cells per mL) was added into a 96-well plate. Gold complexes with concentrations from 0.1–500 μM were dissolved in the culture medium (10 μL) or with 1% DMSO (for **1c**, **2** and **3**), and the solutions were subsequently added into a set of wells. Control wells contained only supplemented media (100 μL). Microtitre plates were incubated at 37 °C in a 5% CO_2/95% air humidified atmosphere for a further 48 h. All the cytotoxcity assays were run in parallel with a negative control (i.e., untreated population) and a positive control with cisplatin as cytotoxic agent.

Assessment of the cytotoxicity was carried out by using a modified Mosmann's method based on the MTT assay. At the end of each incubation period, MTT solutions (10 μL; Cell Proliferation Kit I, Roche) were added into each well, and the cultures were further incubated for 4 h at 37 °C in a 5% CO_2/95% air humidified atmosphere. Then a solubilizing solution (100 μL) was added into the wells to lyse the cells and to solubilize the formazan complex formed. The microtitre plates were maintained in a dark, humidified chamber overnight. The formazan formation was measured with a microtitre-plate reader based on the absorbance at $\lambda =$ 550 nm, and the percentages of cell survival were determined. The cytotoxicity was evaluated based on the percentage cell survival in a dose-dependent manner relative to the negative control.

Inhibition studies of HIV-1 reverse transcriptase by gold(III) complexes: Assays on the in vitro HIV-RT inhibitory activities were conducted by using a commercial assay kit (Reverse Transcriptase Assay, Chemiluminescent, Roche). Complexes **1a–c**, **2**, **3**, $[Zn^{II}(PPIX)]$ and AZT-TP were dissolved in PBS (1.3 μL) or 1% DMSO (for **1c**, **2**, **3** and $[Zn^{II}(PPIX)]$) and mixed with a set of HIV-1 RT in the lysis buffer (2 ng, 128.7 μL) at 37 °C (30 min). A similar procedure was adopted for the combination assay, except that **1a** and AZT–TP were premixed before incubation with the HIV-1 RT solutions. The ELISA assays were conducted by following the manufacturer's instructions. The HIV-1 RT activities were evaluated based on the percentage luminescence of the solutions in a dose-dependent manner relative to the negative control.

Spectroscopic titration for the binding of metalloporphyrins to the WETWWTEYWQ peptide: A solution (1 μM) of the metalloporphyrin (i.e., **1a**, **1c** and $[Zn^{II}(PPIX)]$) in TBS (3000 μL, for **1a**) or with 5% DMSO (for **1c** and $[Zn^{II}(PPIX)]$) was placed in a thermostatic cuvette in a UV/Vis spectrometer, and an absorption spectrum was recorded. Aliquots from a millimolar stock peptide solution were added to the metalloporphyrin solution, and the absorption spectra were recorded after equilibration for 10 min per aliquot until a saturation point was reached. The binding constants (K_b) were determined from the plots of [peptide]/$\Delta\varepsilon_{ap}$ against [peptide],

$$\frac{[\text{peptide}]}{\Delta\varepsilon_{ap}} = \frac{[\text{peptide}]}{\Delta\varepsilon} + \frac{1}{\Delta\varepsilon \times K_b} \quad (1)$$

here $\Delta\varepsilon_{ap} = |\varepsilon_A - \varepsilon_B|$, $\varepsilon_A = A_{obs}/[\text{metalloporphyrin}]$ and $\Delta\varepsilon = |\varepsilon_B - \varepsilon_F|$, with ε_B and ε_F corresponding to the extinction coefficients of peptide-bound and the unbound metalloporphyrin, respectively. By using the absorbance data of the Soret band, the linear plots of [peptide]/$\Delta\varepsilon_{ap}$ versus [peptide] gave the K_b values.

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Keywords: gold · HIV · macrocyclic ligands · porphyrinoids · reverse transcriptase

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