

Supporting Information (SI)

Au(III) compounds as HIV nucleocapsid protein (NCp7)-nucleic acid antagonists.

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Section 1: Experimental Section

Synthesis and Biomolecule Preparation: The Au compounds were prepared as described previously [1,2]. For the MS studies the NC was prepared in the Fabris lab. Briefly, Nucleocapsid (NC) protein of HIV-1 was obtained by *in vitro* expression in *E. coli*, subsequently purified under non-denaturing conditions to preserve the coordination of Zn^{2+} by its characteristic zinc-finger domains. For the gel shift and biophysical studies, the NC (identical sequence) was a generous gift of Dr. R.J. Gorelick, NIH. RNA oligonucleotides corresponding to the SL2 stemloop domain of the HIV-1 genome packaging signal (Ψ -RNA) were purchased from IDT (Coralville, IA) and desalted using ultrafiltration against 150 mM ammonium acetate.

Mass Spectrometry:

Experiments were conducted on a modified Bruker Daltonics (Billerica, MA) Solarix FTICR-MS equipped with a 12T superconducting magnet. Analyses were carried out by direct infusion using tapered quartz nanospray emitters loaded with 5-10 μ L of sample with a spray voltage between 800-1100 V relative to the capillary inlet supplied by an inserted stainless steel wire.

Solutions were prepared as follows:

- A. 1:1 reaction of NC with $[Au(dien)(9-EtGua)]^{3+}$

A solution of $[Au(dien)(9-EtGua)]^{3+}$ (7.5 μ M in 1 μ L H_2O) was added to a solution of NC (7.5 μ M in 1 μ L H_2O) and volume was brought up to 10 μ L with 7 μ L 150 μ M ammonium acetate and 1 μ L isopropyl alcohol. The solution was analyzed immediately in positive ion mode.

- B. 1:1:1 reaction of NCp7/ $[Au(dien)(9-EtGua)]^{3+}$ with SL2-RNA, Figure S1.

A solution of NC (7.5 μ M in 1 μ L H_2O) and $[Au(dien)(9-EtG)]^{3+}$ (7.5 μ M in 1 μ L) was incubated for 30 minutes at room temperature. SL2-RNA (7.5 μ M in 1 μ L), 6 μ L 150 mM ammonium acetate, and 1 μ L isopropyl alcohol were added and the mixture was analyzed immediately in negative ion mode.

- C. Control experiment showing formation of NC/SL2 (2:1) complex, Figure S2

A solution of NC (7.5 μ M in 4 μ L H_2O) was added to a solution of SL2 (7.5 μ M in 2 μ L H_2O) and volume was brought up to 10 μ L with 3 μ L 150 mM ammonium acetate, and 1 μ L isopropyl alcohol. The solution was analyzed immediately. No significant incubation was necessary.

Circular Dichroism Spectroscopy:

Methods were adapted from those previously published [1,3].

Fluorescence Spectroscopy:

Methods were adapted from those previously published [1]. A 3 mL solution of NC (5 μ M) in water was titrated with aliquots of the corresponding quenching compound (7.5 mM) in the range [quencher]/[N-AcTrp] 10-100.

Electromobility Shift Assay

For the NC-SL2 binding control experiments, 32 P end-labeled SL2 hairpin RNA (2nM) was incubated with varying concentrations of NC in binding buffer (50 mM Tris-HCl, 40 mM MgCl₂, 200 mM NaCl, 0.1 mM ZnCl₂, 5% glycerol, and 1% BME) for 30 min. at 30°C. For experiment containing inhibitor, 250 nM NC was incubated with varying concentrations of [Au(dien)(9-EtG)]³⁺ for 30 min. in binding buffer, followed by addition of 32 P-SL2. The reaction was incubated for an additional 30 min. Reactions were separated on a 6% acrylamide gel, dried, and exposed to film.

Fluorescence Polarization

Experiments carried out according to published procedures [4]. For NCp7-SL2 binding control experiments, concentrations of NCp7 (aa 1-55) were mixed with 100 nM 3' fluorescein-labeled hairpin SL2 DNA (sequence GGGGCGACTGGTGAGTACGCCCC) in a final volume of 50 μ l buffer containing 1.25 mM NaCl, 0.125 mM HEPES pH 7.2 in a 96-well black, low-binding microplate (Greiner). FP readings were recorded immediately. For experiments containing inhibitor, varying concentrations of each compound were incubated with 5 μ M NCp7 for 1 h before addition of 100nM SL2 DNA. FP readings were recorded immediately. 5 μ M NC was chosen from the NC-SL2 binding experiment such that 90 % of SL2 was bound. FP readings were recorded immediately.

Efficacy Evaluation in Human Peripheral Blood Mononuclear Cells (PBMCs)

Evaluation was performed by standard SRI procedures. Briefly:

Anti-HIV Efficacy Evaluation in Fresh Human PBMCs

Fresh human PBMCs, seronegative for HIV and HBV, are isolated from screened donors (Biological Specialty Corporation, Colmar, PA). Cells are pelleted/washed 2-3 times by low speed centrifugation and re-suspension in PBS to remove contaminating platelets. The Leukophoresed blood is then diluted 1:1 with Dulbecco's Phosphate Buffered Saline (DPBS) and layered over 14 mL of Lymphocyte Separation Medium (LSM; Cellgro® by Mediatech, Inc.; density 1.078+/-0.002 g/ml; Cat.# 85-072-CL) in a 50 mL centrifuge tube and then centrifuged for 30 minutes at 600 X g. Banded PBMCs are gently aspirated from the resulting interface and subsequently washed 2X with PBS by low speed centrifugation. After the final wash, cells are enumerated by trypan blue exclusion

and re-suspended at 1×10^6 cells/mL in RPMI 1640 supplemented with 15 % Fetal Bovine Serum (FBS), and 2 mM L-glutamine, 4 $\mu\text{g}/\text{mL}$ Phytohemagglutinin (PHA, Sigma). The cells are allowed to incubate for 48-72 hours at 37°C . After incubation, PBMCs are centrifuged and re-suspended in RPMI 1640 with 15% FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin, and 20 U/mL recombinant human IL-2 (R&D Systems, Inc). IL-2 is included in the culture medium to maintain the cell division initiated by the PHA mitogenic stimulation. PBMCs are maintained in this medium at a concentration of $1-2 \times 10^6$ cells/mL with biweekly medium changes until used in the assay protocol. MDMs are depleted from the culture as the result of adherence to the tissue culture flask.

For the standard PBMC assay, PHA stimulated cells from at least two normal donors are pooled (mixed together), diluted in fresh medium to a final concentration of 1×10^6 cells/mL, and plated in the interior wells of a 96 well round bottom microplate at 50 $\mu\text{L}/\text{well}$ (5×10^4 cells/well) in a standard format developed by the Infectious Disease Research department of Southern Research Institute. Pooling (mixing) of mononuclear cells from more than one donor is used to minimize the variability observed between individual donors, which results from quantitative and qualitative differences in HIV infection and overall response to the PHA and IL-2 of primary lymphocyte populations. Each plate contains virus/cell control wells (cells plus virus), experimental wells (drug plus cells plus virus) and compound control wells (drug plus media without cells, necessary for MTS monitoring of cytotoxicity). In this *in vitro* assay, PBMC viability remains high throughout the duration of the incubation period. Therefore, infected wells are used in the assessment of both antiviral activity and cytotoxicity. Test drug dilutions are prepared at a 2X concentration in microtiter tubes and 100 μL of each concentration (nine total concentrations) are placed in appropriate wells using the standard format. 50 μL of a predetermined dilution of virus stock is placed in each test well (final MOI $\cong 0.1$). The PBMC cultures are maintained for seven days following infection at 37°C , 5% CO_2 . After this period, cell-free supernatant samples are collected for analysis of reverse transcriptase activity and/or p24 antigen content. Following removal of supernatant samples, compound cytotoxicity is measured by addition of MTS to the plates for determination of cell viability. Wells are also examined microscopically and any abnormalities are noted.

Data Analysis

Using an in-house computer program, IC_{50} (50% inhibition of virus replication), IC_{90} (90% inhibition of virus replication), IC_{95} (95% inhibition of virus replication), TC_{50} (50% cytotoxicity), TC_{90} (90% cytotoxicity), TC_{95} (95% cytotoxicity) and therapeutic index values ($\text{TI} = \text{TC}/\text{IC}$; also referred to as Antiviral Index or AI) are provided. Raw data for both antiviral activity and toxicity with a graphical representation of the data are provided in a printout summarizing the individual compound activity.

Note Concerning Use of Human Samples: All human materials used in support of the work described in this manuscript was acquired in full compliance with applicable State and Local laws and the provisions of the Uniform Anatomical Gift Act in the United States, and no undue inducements, monetary or otherwise, were offered to any person to influence their donation of human material. The work described in this manuscript included the use of fresh human peripheral blood mononuclear cells. The human blood used for isolation of these cells at Southern Research Institute was purchased commercially from vendors (*e.g.*, Biological Specialty Corporation, Colmar, PA) that provide blood products collected from healthy volunteer donors. As such, there are no links that can be used to identify the subjects from whom the specimens were obtained, either directly or indirectly, through coding systems. Therefore, the HHS human subjects regulations (45 CFR Part 46) do not apply to the use of these cells in the described research. The human subjects regulations decision charts from the Office of Human Research Protection (OHRP) were used to determine whether the described studies fall under the human subjects regulations and if so, whether a research project met the criteria for Exemption 4.

References:

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3. de Paula, Q. A.; Mangrum, J. B.; Farrell, N.P. *J. Inorg. Biochem.* 2009, *103*, 1347-1354.
4. Breuer S, Chang MW, Yuan J, Torbett BE. *J. Med. Chem.* 2012 *55*, 4968-4977.

Section 2. Supplementary Figures

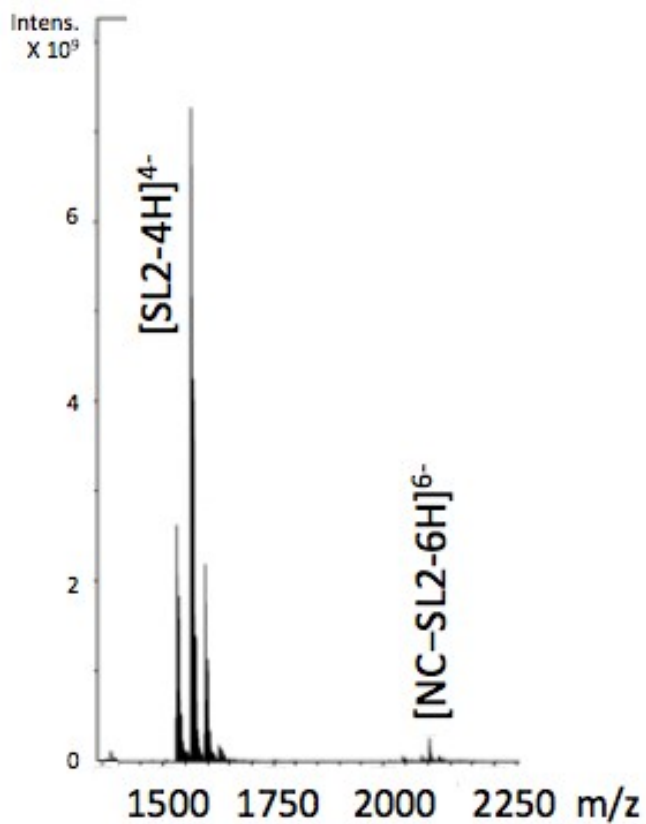


Figure S1. FT-ICRMS spectrum (negative ion mode) of 1:1 [Au(dien)(9-EtGua)]³⁺ / NC mixture (incubated for 30 minutes) followed by addition of SL2 RNA.

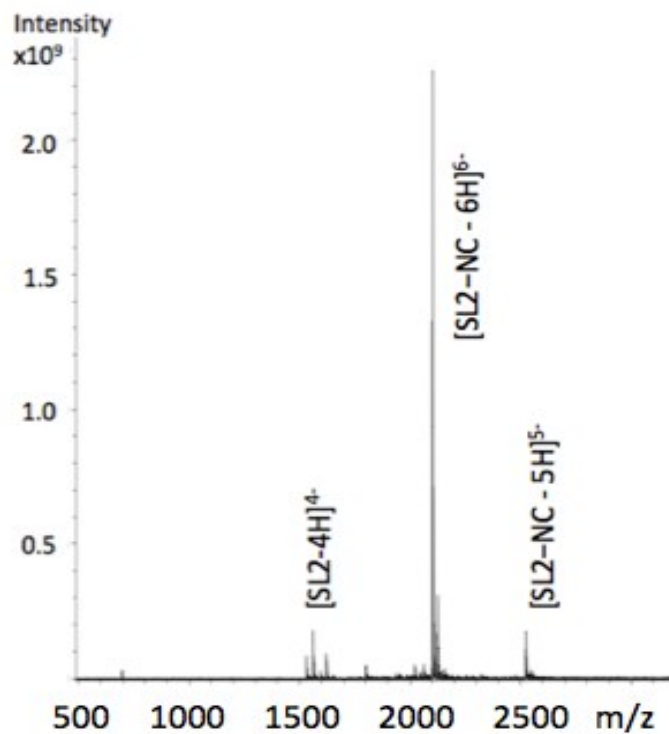


Figure S2. FT-ICRMS spectrum (negative ion mode) of NC:SL2 RNA complex in a 2:1 ratio. Note that under these MS conditions, no free RNA is present and the complex remains intact. These conditions were used before addition of any metallated nucleobase.

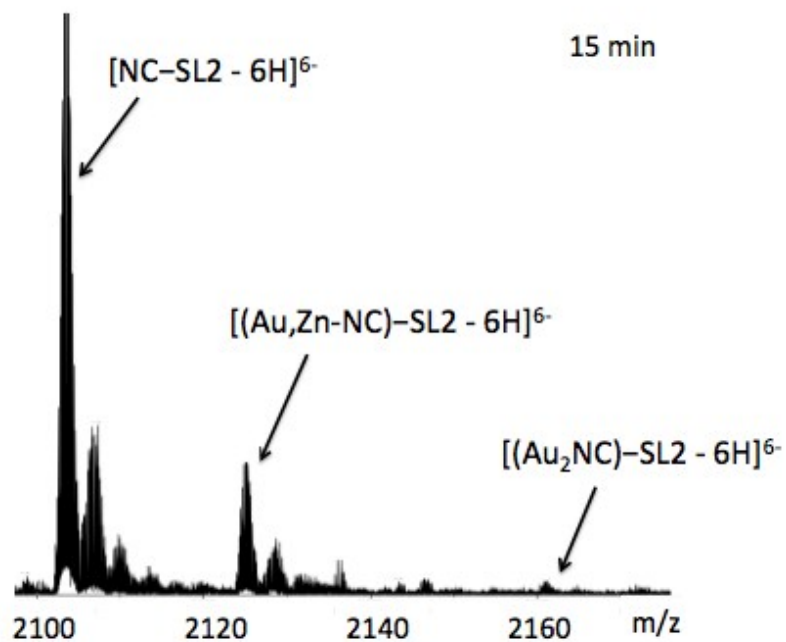


Figure S3. Expanded region of 2:1 NC/RNA complex reacted with 1 eq. $[\text{Au}(\text{dien})(9\text{-EtGua})]^{3+}$ showing presence of possible RNA-Au-NC crosslinking.

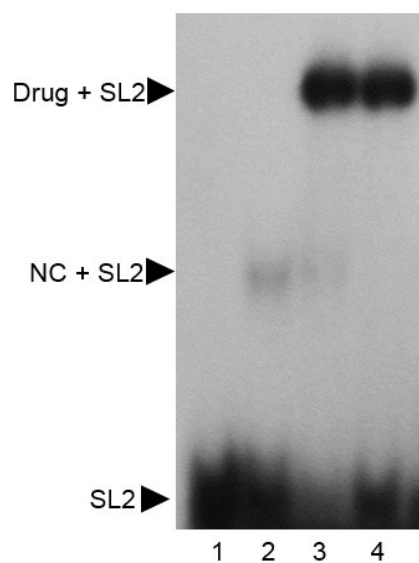


Figure S4. Identification of drug-SL2 aggregates. Lane 1, SL2 only; Lanes 2, NC was incubated with SL2 for 30 min.; Lane 3, NC was incubated with drug for 30 min., followed by SL2 for an additional 30 min.; Lane 4, drug was incubated with SL2 for 30 min.

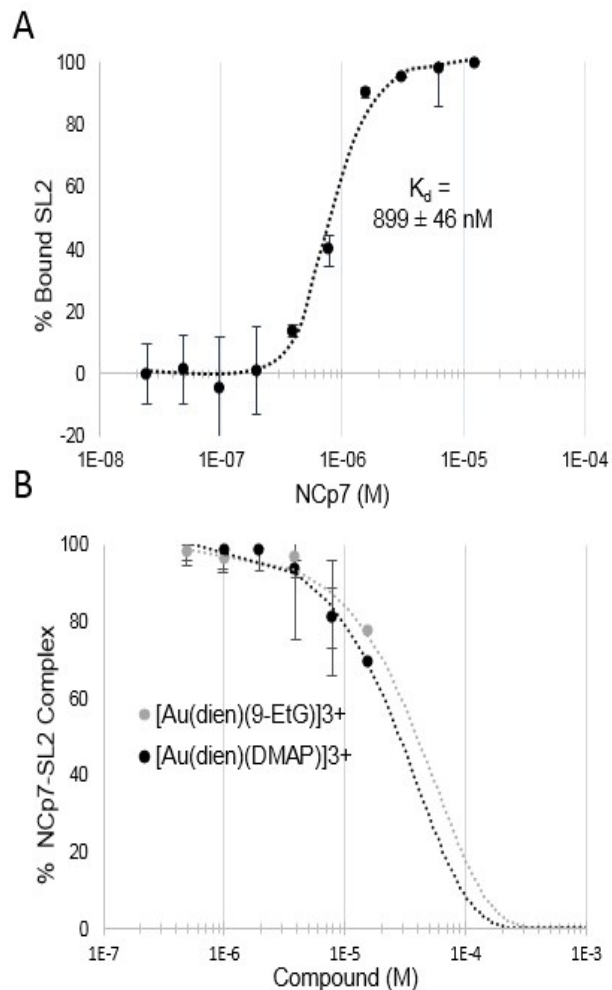


Figure S5. $[\text{Au}(\text{dien})(9\text{-EtGua})]^{3+}$ and $[\text{Au}(\text{dien})(\text{DMAP})]^{3+}$ inhibit SL2-NC binding. A. Fluorescence polarization control experiment showing high affinity binding of NC to SL2. B. $[\text{Au}(\text{dien})(9\text{-EtGua})]^{3+}$ and $[\text{Au}(\text{dien})(\text{DMAP})]^{3+}$ displacement of SL2 from NCp7-SL2 complex. Higher concentrations of drug could not be interpreted due to drug-RNA aggregation. The data shown represents the average of two independent experiments performed in duplicate.

Table S1. *In Vitro* Inhibition of HIV Infectivity of Au Compounds.

Compound	Virus	High Test Concentration	IC ₉₀ (μM)	IC ₅₀ (μM)	TC ₅₀ (μM)	TI (TC ₅₀ /IC ₅₀)
Auranofin	Ba-L ^a	100 μM	0.75	0.15	1.72	11.6
	NL4-3 ^b		0.97	0.20		8.44
	91US001 ^c		0.81	0.24		7.29
[Au(dien)(9-EtG)]	Ba-L	100 μM	99.1	38.3	> 100	> 2.61
	NL4-3		82.5	19.1		> 5.24
	91US001		98.6	46.5		> 2.15
[Au(dien)(DMAP)]	Ba-L	100 μM	92.5	27.6	> 100	> 3.62
	NL4-3		75.1	19.2		> 5.20
	91US001		73.9	20.3		> 4.93
AZT	Ba-L	1,000 nM	51.7 nM	2.65 nM	> 1,000 nM	> 377
	NL4-3		13.7 nM	5.04 nM		> 199
	91US001		28.4 nM	1.63 nM		> 614

IC₅₀/IC₉₀: Concentration to inhibit infectivity. TC₅₀: Cytotoxicity. TI: Therapeutic (Selectivity) Index.

^aCCR5-tropic, Group M Subtype B, lab-adapted isolate

^bCXCR4-tropic, Group M Subtype B, molecular clone

^cCCR5-tropic, Group M Subtype B, clinical isolate