Mechanism of Multivalent Nanoparticle Encounter with HIV-1 for Potency Enhancement of Peptide Triazole Virus Inactivation

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**Background:** HIV-1 envelope spike protein remains a compelling but elusive target for preventing infection. A peptide triazole (PT) class of entry inhibitor has previously been shown to bind to HIV-1 gp120, suppress interactions of the Env protein at host cell receptor binding sites, inhibit cell infection and cause envelope spike protein breakdown, including gp120 shedding and, for some variants, virus membrane lysis. We found that gold nanoparticle conjugated forms of peptide triazoles (AuNP-PT) exhibit substantially more potent anti-viral effects against HIV-1 than corresponding peptide triazoles alone. Here, we sought to reveal the mechanism of potency enhancement underlying nanoparticle conjugate function. We found that altering the physical properties of the nanoparticle conjugate, by increasing either or both the AuNP diameter and the density of PT conjugated on the AuNP surface, enhanced potency of infection inhibition to impressive picomolar levels. Further, compared to unconjugated PT, AuNP-PT was less susceptible to reduction of anti-viral potency when the density of PT-competent Env spikes on the virus was reduced by incorporating a peptide-resistant mutant gp120. We conclude that potency enhancement of virolytic activity and corresponding irreversible HIV-1 inactivation of PTs upon AuNP conjugation derives from multivalent contact between the nanoconjugates and metastable Env spikes on the HIV-1 virus. The findings reveal that multi-spike engagement can exploit the metastability built into virus envelope to irreversibly inactivate HIV-1, and provide a conceptual platform to design nanoparticle-based antiviral agents, for HIV-1 specifically and putatively for metastable enveloped viruses generally.
INTRODUCTION

There is an urgent need for new antiretroviral agents for the prevention and treatment of HIV-1 infection. Current inhibitors used in most combination antiretroviral therapies target the viral enzymes reverse transcriptase, integrase and protease. Although the use of combination antiretroviral therapy has resulted in reducing viremia and HIV-1 related morbidity and mortality (1, 2), these drugs target stages of HIV-1 infection after cell entry and have significant challenges such as complex regimens and treatment-resistant variants of HIV-1. Currently, there are only two FDA approved antiretroviral drugs targeting the initial entry of the virus into host cells, namely T20 (enfuvirtide) and maraviroc (3-8), but these are used mainly in salvage therapy due to such limitations as injection site reaction and cost of production for the former, and incomplete co-receptor breadth for the latter (7, 9-11). T20, a fusion inhibitor, targets transient conformations of the virus Env protein, gp41, and hence has a relatively short time window to act against the HIV-1 host cell membrane fusion process (12, 13). Maraviroc, on the other hand, targets the host cell co-receptor CCR5 and can only block R5-tropic HIV-1 virions from infecting the cell (7, 11, 14). Therefore, prolonged maraviroc treatment can result in the development of X4-tropic HIV-1 (7, 11, 14).

Host cell infection by HIV-1 is mediated by cell receptor interactions with trimeric envelope glycoprotein (Env) spikes that are exposed on the virus membrane surface (15, 16). Env is the only virally encoded protein on the virion surface (16, 17). The external gp120 component of Env is essential for cell receptor (CD4) and coreceptor (usually CCR5 or CXCR4) interactions and subsequent virus-cell fusion (17, 18). Hence, Env gp120 presents an obvious target to attack the virus directly in order to block the cascade of integrated binding and conformational change steps that lead to host cell infection. Env-specific inhibitors that could inactivate the virus before receptor encounter would hold great promise in preventing AIDS transmission and progression. In spite of the potential of gp120 antagonists for prevention and intervention of infection, progress has been limited for such agents, due to such factors as high rate of mutation of gp120 protein leading to the threat of resistance to developing inhibitors (19-21), low potency of small molecule inhibitors (22-24), the high cost and potential toxicity of protein inhibitors (25-27), and the potential risk of infection enhancement with CD4-mimicking ligands (22, 28).

Previously we showed that a family of entry inhibitors, peptide triazoles (29-34) derived from the peptide 12p1 (35, 36) bind specifically and with high affinity to HIV-1 Env gp120, antagonize the interactions of Env binding sites for both host cell receptors CD4 and CCR5/CXCR4 and cause gp120 shedding from the virus surface, leading to HIV-1 inactivation before host cell encounter (37, 38). The most potent compositions of peptide triazole inactivators discovered so far are multivalent forms conjugated to monodisperse gold nanoparticles. The conjugates initially derived were found to have low nanomolar inhibitory IC50 values and to act as virucidal agents leading to the irreversible breakdown of the virus before the virus has a chance to engage the host cell (37, 38).

The functional properties of virolytic gold nanoparticle-peptide triazole (AuNP-PT) conjugates make it feasible for long term use of this type of agent to inactivate HIV-1 before host cell encounter. In the current work, we sought to investigate the mechanism of cell-free HIV-1 inactivation using the AuNP-PT multivalent nanostructures. We evaluated the impact of [1] altering the properties of the AuNP-PT constructs, by increasing size and PT coverage density of the nanoparticles, and [2] varying the HIV-1 virion functional spike density. Our investigations demonstrate the importance of multivalent contact between nanoparticle and metastable virus interaction partners. The results define Env-targeting NP designs that take advantage of HIV-1 Env metastability and cause specific and irreversible HIV-1 inactivation. Overall, the investigation demonstrates that the multivalent physical contact approach can be used as a general strategy for antagonizing HIV-1 transmission and proliferation.
EXPERIMENTAL MATERIALS AND METHODS

Materials

For peptide triazole synthesis, all FMOC-protected α- and β-amino acids, O-benzotriazole-\(N,N,N',N'\)-tetramethyl-uronium-hexafluoro-phosphate (HBTU), 1-hydroxybenzotriazole (HOBt), Rink amide resin (4-(2',4'-Dimethoxyphenyl-FMOC-amominethyl)phenoxy resin) with a 0.55 mmol/g substitution, \(N,N\)-dimethylformamide (DMF), pyridine and \(N,N\)-di-isopropylethylamine (DIPEA) were purchased from Chem-Impex International Inc. Ethynylferrocene and Cu(I)I were purchased from Sigma-Aldrich and used without further purification. FMOC-cis-4-azidoproline was synthesized starting with commercially available trans-hydroxyproline-OH (31). Gold (III) chloride hydrate and citric acid were obtained from Sigma-Aldrich, Bis (\(p\)-sulfonatophenyl) phenylphosphine dehydrate dipotassium salt (BSPP) from Strem Chemicals. Citric acid and cysteine used in the conjugation were from Sigma Aldrich. Modified human osteosarcoma cells (HOS.T4.R5) engineered to express CD4 receptor and CCR5 co-receptor, receptor and co-receptor respectively, as well as pNL4-3 Luc+ R-E-backbone DNA, were obtained from Dr. Nathaniel Landau. The HOS.T4.R5 cells were grown in DMEM supplemented with 10% FBS, 2.5% HEPES, 1% Penicillin- Streptomycin, 2% L-Glutamine and 1 mg of puromycin. 293T cells were obtained from American Type Culture Collection and grown in the same culture medium as the HOS.T4.R5 cells except without puromycin. The plasmids for HIV-1 BaL-01 gp160 and VSV-G Env DNA were obtained from the NIH AIDS Reagent Program. Replication competent HIV-1 BaL-01 virions were obtained from the University of Pennsylvania Center for AIDS Research (Penn CFAR). Mouse anti-p24 and rabbit anti-p24 antibodies, and p24 protein, were purchased from Abcam. Fully Infectious HIV-1 (BaL) was a gift from Dr. Michele Kutzler and obtained from the Penn CFAR. The gp120 monomer protein was produced using an already-established protocol (33), anti-gp120 D7324 was purchased from Alto chemicals. Gp41 protein, Enfurvirtide (T20) and anti-gp41 antibodies 4E10 and 2F5 were obtained from the NIH AIDS Reagent Program. Enhanced Chemiluminescence western blot detection system was obtained from Amersham. O-phenylenediamine (OPD) was from Sigma Aldrich. All other materials were purchased from Fisher Scientific.

Peptide triazole synthesis: To conjugate peptide triazole on nanoparticles, the peptide triazole KR13 (RINNIXWSEAMMβAQβACamide, X=ferrocenyltriazolePro) was synthesized to contain the 12-residue N-terminal sequence of the HNG-156 parent peptide (RINNIXWSEAMMamide) (39) with a C-terminal extension containing a free thiol group. KR13 itself contained the essential peptide triazole pharmacophore, Ile-triazole-Pro-Trp, and was functionally active (37). The free sulfhydryl group was incorporated to enable direct thiol conjugation to the gold surface of AuNP (38). The peptide KR13 binds to gp120 with a \(K_D\) of 11.3 nM (37).

Gold nanoparticle synthesis: The citrate reduction method developed by Frens (40) was modified in order to synthesize size-controlled, stable and monodisperse AuNPs. The citrate acid concentration was varied to obtain AuNP with various sizes ranging from 13 nm – 123 nm. The citrate reaction solution, initially at 100 °C, was cooled to room temperature and BSPP (18 mg/ml for 20 nm AuNP) was added to the synthesized particles and stirred overnight at room temperature. The resulting particles were further washed with phosphate buffer at pH 7.2 and concentrated using a Millipore 100,000 kDa filter. The particle size was determined by dynamic light scattering (DLS) in the Zetasizer NS90 (Malvern Instruments), and the particle concentration was calculated using the absorbance at 450 nm and the peak absorbance of the UV spectrum (41). The AuNP particle morphology was characterized using transmission electron microscopy. Samples were prepared by adding a drop of the AuNP solution onto a lacy carbon grid film and then allowing evaporation. TEM images of AuNPs were taken with a JEM 2100 camera operated at 200 kV.

Gold nanoparticle-peptide triazole conjugate production: Peptide conjugation to the AuNPs was achieved via covalent linkage between the thiol moiety (-SH) on C-terminal cysteine residue and the gold surface of AuNP (38). A flocculation assay (42) was conducted to determine
the ratio of AuNP to KR13 to use for complete peptide conjugation without peptide aggregation. The BSPP-stabilized AuNP-KR13 mixtures in phosphate buffer were stirred vigorously for 30 minutes at 25°C. Excess peptide was removed from the nanoparticle fraction using a 10,000 MW cut-off centrifugation filter. PT content of AuNP-KR13 preparation was determined using amino acid analysis after acid hydrolysis (Keck Institute, Yale University). To prepare AuNP-PT particles with varying density of KR13, we used 20 nm AuNPs. These were mixed with different molar ratios of KR13 and L-cysteine during the step of peptide conjugation to AuNP. Both molecules present a single reactive thiol for covalent attachment to the BSPP-stabilized nanoparticle. However, AuNP-Cys has no virolytic activity (Table 2), and only the KR13 component will bind to HIV Env on the virion surface. Incorporation of KR13 was measured directly by amino acid analysis. We calculated the average areal peptide density (ρ) assuming spherical NP’s,

\[ \rho = \frac{C_{\text{peptide}}}{C_{\text{AuNP}} \pi d^2} \]

Where \( \pi d^2 \) is surface area of nanoparticle and \( C_{\text{KR13}}/C_{\text{AuNP}} \) is the molar ratio of peptides per nanoparticle. The conjugates were validated for peptide conjugation by testing their change in zeta potential, which was measured by the Zetasizer NS90 (Malvern Instruments) (data not shown). Furthermore the monodispersity was assessed using both DLS (Figure 1B) and TEM (Figure 1C).

**HIV-1 pseudotyped virion production:**
The recombinant virus consisted of the pro-viral envelope plasmid sequence corresponding to the CCR5 targeting HIV-1BaL strain or a VSV (Vesicular Stomatitis Virus) pseudotype, and the backbone sequence corresponded to an envelope-deficient pNL4-3 luc+ env+ provirus developed by N. Landau (43, 44). Four µg of envelope DNA and eight µg of backbone DNA were co-transfected into 293T cells using FuGene 6 as the transfection reagent (Promega). Fourteen hours post-transfection, the medium was changed, and the VLP containing supernatants were collected at 48 hours post transfection. Soluble proteins (including Env gp120 and p24) from the viral supernatants were removed via fractionation on 6-20% iodixanol gradient for 2 hours at 210,700 x g (SW41 rotor, Beckman ultracentrifuge). The collected fractions were validated for p24 content using capture ELISA as well as gp120 content using the western blot detection. Virions purified on the 6-20% iodixanol gradient exhibited a characteristic distribution profile of p24 and gp120 content, enabling viral fractions (18-19.2% iodixanol) and soluble protein fractions (6-8% iodixanol) to be isolated. The gradient-purified virus samples, which exhibited full or greater infectivity (against HOS.T4.R5 cells (38)) compared to the unfraccionated control virions, were collected, aliquoted and stored at 80°C until further use.

**Env spike presentation on the virus surface:** To make viruses with varying spike density, HEK293T cells were transfected with backbone vector, pNL4-3.Luc R-E-, and a mixture of active Env plasmid (HIV-1 BaL - WT) with an Env plasmid encoding inactive Env gp120 S375W BaL. The S375W mutation has been found previously to be fusion competent (45, 46) but it does not bind significantly to KR13 and hence causes resistance to PT (36, 47). Of note, varying density does not in itself eliminate the potential for local clustering, and indeed evidence has been obtained showing that HIV-1 Env spikes have the tendency to cluster (48, 49). Control virions included those with all-[BaL or S375W Env] (all-active or resistant for peptide triazole binding respectively). Protease digestion of the spike varying virion was conducted in order to eliminate non-functional envelopes. This digestion of pseudoviruses was carried out by treating the culture supernatants with a protease cocktail of 1 µg of trypsin, chymotrypsin, subtilisin, and/or proteinase K (Sigma) at 37°C as described by Crooks et al. (50). The treated virions were spun on a 6-20% iodixanol gradient as described above. Spike density was quantified using western blot analysis of gp120, viral infection and p24 content (ELISA) as explained above (data not shown). As expected, the S375W mutant was similar to the wild type BaL in infecting the HOS.T4.R5 cells (data not shown).

**Anti-viral functions of AuNP-KR13 conjugates**

**Dependence of anti-viral effects on the size of AuNP-KR13:** To test the effects of
nanoparticle size on viral inhibition and virolytic activity, we synthesized AuNPs with diameters ranging from 10-200 nm as previously described and functionalized them with the KR13 peptide. We utilized assays for HIV cell infectivity and for virus contents, including p24 and gp120, in order to correlate nanoparticle diameter and surface area of the AuNP-KR13’s with antiviral effects. Purified virus was treated with AuNP-KR13 constructions for thirty minutes at 37°C and spun on a 6-20% iodixanol gradient for 2 hours at 210,700 x g (ultracentrifugation as above). The collected virus fraction and the supernatant fraction were tested for infectivity, p24 and gp120 content post treatment. Virus treated with PBS for 30 minutes at 37°C was used as negative control for background signal, and virus incubated for 5 minutes with 2% Triton-X at 98°C was the positive control for p24 and gp120 content.

**Modulating nanoparticle surface density of KR13 on AuNP:** KR13 coverage on 20 nm diameter AuNP was varied by mixing different molar ratios of KR13 and L-cysteine during the step of KR13 conjugation to AuNP. Both molecules present a single reactive thiol for covalent attachment to the BS3-stabilized nanoparticle. However, only the KR13 was expected to bind to HIV envelope glycoproteins on the surface of the virion. AuNP and KR13 were synthesized and functionalized as described above. Cysteine/KR13 molar ratios were varied from 0:1000 to 1000:0 in increments of 50. We calculated the density of peptide coverage (ρ) as explained above. Purified virus was treated with AuNP-KR13 constructions for thirty minutes at 37°C and spun on a 6-20% iodixanol gradient for 2 hours at 210,700 x g (ultracentrifugation as above) (37). The collected virus fraction and the supernatant fraction were tested for infectivity and both p24 and gp120 contents post treatment.

**AuNP-KR13 effects on replication competent HIV-1:** Replication competent HIV-1 BaL virions were obtained from the Penn CFAR, purified using 6-20% iodixanol gradient and p24 content measured using ELISA. The inhibitor concentration that gives 50% reduction of the viral titer in tissue culture (IC₅₀) was determined from dose response data using nonlinear least-squares regression analysis with Origin V.8.1 (Origin Lab). The working dilution of virus was pre-treated with a serial dilution of AuNP-KR13 (20 nm and 80 nm), starting from an effective KR13 concentration on the AuNP surface of 50 µM and 10 µM respectively, for 30 min at 37 °C. HOS.T4.R5 cells, seeded at 8,000 cells per well, were incubated for 24 hours followed by addition of pre-incubated inhibitor-virus mixtures. After 48 hours infection, with a growth medium wash step 24 hours post treatment, the p24 content of virions in the supernatant was measured in order to determine the IC₅₀ value of inhibition of virus infection. For cell-free p24 release experiments, the peptide triazole and cell-free replication competent HIV-1 BaL virus were pre-incubated for 30 min at 37°C followed by spinning for 2 hours at 23,000 x g in a table top centrifuge (Eppendorf) at 4°C. Supernatant was separated and p24 content determined using the p24 capture ELISA as described above. The extents of viral inhibition of, and p24 release from, fully infectious HIV-1 BaL were also evaluated for the free control peptide KR13 (data from Bastian et al. 2013) (37).

**Comparisons of anti-viral functions of AuNP-KR13 to KR13 alone**

**Exposure of gp41 in viruses treated with AuNP-KR13 and KR13:** The HIV-1 BaL pseudotype was incubated with increasing concentration of either KR13 starting at 50µM or AuNP-KR13 starting at 1 µM (KR13 concentration) for 30 minutes at 37°C. The samples were spun at 23,000 x g (table top centrifuge) for 2 hours at 4°C, and the virion fraction for each sample was collected and fixed using a homemade fixative with 0.1% formaldehyde and 0.1% gluteraldehyde in PBS. Glycine (0.1 M) was added to stop the crosslinking reaction by the fixative. The virions then were loaded onto an ELISA plate (high protein binding) and incubated overnight on a shaker at 4°C. The plate was blocked with 3% BSA, and ELISA was used to detect gp41 epitopes with human gp41 antibodies 2F5 and 4E10 followed by addition of anti-human IgG HRP secondary antibody.

**Effect of fusion inhibitor enfurvirtide (T20) on virolysis induced by AuNP-KR13 and KR13:** T20 was shown previously to effect the 6-helix bundle formation of the HIV-1 virion during
fusion with the host cell (8, 13). Further, T20 was found to specifically inhibit KR13-induced HIV-1 cell-free virolysis (37). A corresponding experiment was conducted to test whether T20 inhibited virolysis of pseudotyped HIV-1 BaL induced by AuNP-KR13 (20.2 nm diameter of AuNP). The AuNP-KR13 was kept at an IC_{50} concentration of 25 nM, and serial dilutions of T20 starting at 1 µM were co-incubated with pseudotyped HIV-1 BaL for 30 min at 37°C. Treated virion samples were fractionated on a 6-20% iodixanol gradient (above). Gradient fractions were quantified for p24 using ELISA, and relative p24 release was quantified and plotted using Origin V.8.1 (Origin Lab).

**Time dependence of AuNP-KR13 induced HIV-1 breakdown:** The time dependence of gp120 shedding, p24 release and inhibition of pseudotyped HIV-1 BaL by an IC_{50} concentration of 20.2 nm AuNP-KR13 (25 nM) was measured. The AuNP-KR13 was incubated with the virus for different times ranging from 1 min to 24 hours at 37°C, the samples were spun on a 6-20% iodixanol gradient and the gradient fractions were tested for gp120 shedding, p24 release and inhibition of viral infection. For gp120 shedding, the fractions were tested using a western blot analysis with primary anti-gp120 antibody, D7324, and secondary antibody, anti-sheep HRP. For p24 release, the ELISA explained above was used. The western blots were analyzed using Image J western blot band quantification. For inhibition of viral infection, the luciferase reporter assay was used as explained above (37).

**Anti-viral effects of AuNP-KR13 vs. KR13 on HIV-1 pseudovirions with modified Env spike presentation on the virus surface:** Comparisons were made of the lytic deformation of viruses by KR13 and AuNP-KR13 with viruses containing a varying proportion of KR13-resistant mutant vs. WT Env. In the western blot analysis, the amount of loaded virions was normalized using both p24 content from ELISA and total protein content measured using Bradford assay. The p24 contents of the different virions and their total protein contents were similar, within ± 1.5%. Thus, virus amounts were determined by p24 content (3000 molecules per virion (51, 52)). Antiviral assays included inhibition of infection by KR13 compared to AuNP-KR13 on HOS.T4.R5 cells, and cell-free HIV-1 p24 release.

**Morphological analysis of HIV-1 after AuNP-KR13 conjugate treatments**

Transmission electron microscopy (TEM) was conducted to visualize the morphology of the virions treated with AuNP-KR13 (20.2 nm and 80.2 nm diameter AuNP). Purified pseudotyped HIV-1 BaL virus and AuNP-KR13 were pre-incubated from 5-1440 min at 37°C. Following incubation, samples were fixed with 2% glutaraldehyde for 30 minutes at room temperature, and then embedded in Spurr’s low viscosity epoxy medium after acetone washes to dry the virions. Slices (100 nm thick) were prepared using an ultra-microtome (Leica EM UC6), loaded onto a holey carbon TEM 200 mesh grid (Electron Microscopy Science) and imaged using the JEM 2100 camera operated at 120 kV (JEOL, Japan). Sixteen images were taken per sample, and the sizes of observed particles were determined, using Image J software to derive average diameters of the virion particles from TEM images measured from 5 angles. In order to confirm identification of collapsed virions in samples imaged, we used EDAX (Energy Dispersive Spectroscopy) and related the element ratios on untreated virions to those of the collapsed particles.

**RESULTS**

**Monodispersity of gold nanoparticle-peptide triazole conjugates**

In this study, we investigated the mechanism underlying the antiviral functions of AuNP-KR13 conjugates against HIV-1, and in particular the ability of the nanoconjugates to cause specific virus lytic inactivation. To achieve this, the dependence of lysis on variations of surface characteristics, of both nanoparticle inhibitor and virion envelope protein, was measured.

The physical characteristics of gold nanoparticle-peptide triazole conjugates were validated by DLS, TEM and UV absorbance. Particle size was obtained by DLS using the Zetasizer NS90 (Malvern Instruments), while the particle concentration was calculated using the UV
spectrum as described in the methods section (41). The total number of peptide molecules per AuNP was obtained by amino acid analysis. The DLS data are shown in Figure 1, along with representative TEM images of AuNP-KR13 conjugates of various AuNP sizes. Figure 1A also shows the comparison between the expected sizes of AuNP using varying concentrations of citric acid as well as the experimentally obtained diameters (DLS). The AuNP-KR13 conjugates showed good monodispersity as observed by the TEM images in Figure 1C. Further, the zeta potential values for AuNP-KR13 conjugates compared to the AuNP alone verified the successful peptide conjugation to AuNP (data not shown) (53).

Anti-viral effects of AuNP-KR13 conjugates

Relationship between AuNP-KR13 particle size and antiviral activity: AuNP-KR13 conjugates were tested for both inhibition of HIV-1 BaL pseudotype infection of HOS.T4.R5 cells as well as cell-free p24 release, in both cases after 30 min exposure of viruses to AuNP-KR13 conjugates. Figure 2 shows the dose responses of the effects, and IC50 values are summarized in Table 1. Strikingly, the anti-viral effects were greatly enhanced with increasing size of the nanoparticle. This effect is likely due to decreasing nanoparticle curvature and corresponding increasing contact area between the AuNP-KR13 nanoparticle and the HIV-1 virion. Table 1 also shows the number of peptides per nanoparticle calculated from amino acid analysis and the KR13 areal density on the AuNP surface.

To control for differences in peptide density on separate populations of AuNPs, we prepared AuNPs of 20.2 nm, 42.3 nm and 80.2 nm diameters with nearly equal peptide densities by empirically adjusting the peptide concentration used during conjugation. The antiviral effects observed upon size variation of AuNP at (nearly) fixed KR13 areal density in AuNP-KR13 conjugates are shown in Figure 3, and the IC50 values obtained from the dose response profiles are given in Table 2. Here, a clear correlation was observed between the effects of AuNP size on inhibition of viral infection and p24 release, with increasing size leading to increasing potency. This result suggests that the AuNP-KR13 induced p24 release from HIV-1 is related to the extent of contact of the HIV-1 virion surface with multiple KR13 monomers on the nanoparticle surface. In retrospect, the more limited increase in p24 release potency initially observed with nanoparticle size increase in initial experiments (Figure 2) likely was due to the fact that the densities of KR13 per unit area on the AuNP surface decreased with increasing size (Table 1), while, in Figure 3B, increasing size of AuNP, while maintaining a similar density of KR13 on the AuNP surface, caused a drastic increase in the potency of p24 release from HIV-1.

Impact of density variation of peptide triazoles conjugated to AuNP on anti-viral functions: AuNP-KR13 conjugates were prepared with varying KR13 densities on 20 nm nanoparticles and tested for both inhibition of HIV-1 BaL pseudotype cell infection with HOS.T4.R5 cells and cell-free p24 release after 30 min of exposure of virus with the conjugates. The values computed for KR13 coverage per square nanometer of the AuNP surface assumed that AuNP is a perfect sphere and that individual KR13’s are randomly distributed on the NP surface. As shown in Figure 4 and Table 3, both viral infection inhibition and p24 release activities were greater with increasing areal density of KR13 on the AuNP surface. The AuNP particles without KR13 had 100% cysteine coverage and did not exhibit non-specific anti-viral effects (Table 3 and Figure 4).

AuNP-KR13 potencies with replication competent HIV-1: The anti-viral effects of 20 and 80 nm AuNP-KR13 particles were tested with replication-competent Bal-01 HIV-1, as shown in Figure 5. The results demonstrate that the AuNP-KR13 is effective and has a greater potency than KR13 alone (approximately 300 fold greater for the 20 nm AuNP-KR13 and 1400 fold greater for the 80 nm AuNP-KR13 compared to KR13 (37)).

Comparison of AuNP-KR13 and KR13 actions on pseudoviruses

We previously observed that KR13-induced lysis of HIV-1 embodies virus breakdown characteristics reminiscent of those occurring during virus-cell fusion. These include exposure of the membrane proximal external region (MPER) of gp41 and inhibition by fusion inhibitor T20. In the current
work, we sought to evaluate these characteristics for AuNP-KR13 lysis. As shown in Figure 6A, AuNP-KR13 treatment did not induce a concentration-dependent exposure of MPER on the residual virion, while treatment with KR13 did. In addition, as seen in Figure 6B, the fusion inhibitor, T20, did not lead to the inhibition of AuNP-KR13 induced p24 release, in contrast to the inhibition seen with KR13. Figure 6C shows that, like KR13, AuNP-KR13 induced virolysis was accompanied by gp120 shedding. However, for AuNP-KR13, gp120 shedding, p24 release and viral infection inhibition occurred simultaneously, in all cases starting within 10 min of incubation with the virus. By comparison, in KR13 virolysis, p24 release occurred with a time lag compared to the gp120 shedding and virus infection inhibition as shown in Bastian et al. 2013 (37). Hence, from the data shown above, the AuNP-KR13 induced virolysis process appears to be controlled differently than that of KR13. In order to further compare AuNP-KR13 with KR13, we turned our attention to the surface properties of the virus.

Impact of Env trimer composition on the antiviral potencies of peptide-triazole inhibitors

The enhanced antiviral activity of AuNP-KR13 compared to KR13 suggests that multivalent interactions between the virion and nanoparticle are important determinants of AuNP-KR13 potency. Such interactions could involve multivalent attachment to single Env trimers and/or localized trimer cross-linking. To further probe the role of multivalency in inhibition, we compared the antiviral activities of AuNP-KR13 and KR13 against HIV-1 pseudotyped with both susceptible and non-susceptible BaL Env variants. The non-susceptible Env contained the gp120 S375W mutation that ablates peptide-triazole binding, by steric blockade of the PT Trp indole side chain (36, 47), but has negligible impact on glycoprotein expression, processing or fusogenic activity (data not shown). Viruses were produced from cells co-expressing WT (denoted W) and S375W (denoted M) Env at different relative levels. The resulting viral populations shared the same total Env content (W + M) but varied in the average surface density of WT Env. When co-expressed in virus producing cells, WT and S375W Env form four trimeric species (W3, W2M, WM2 and M3), the distribution among which can be approximated through binomial statistics assuming the protomers assort randomly:

$$\alpha_i = \frac{3!}{i!(3-i)!} f^i (1 - f)^{3-i} \quad \text{…………………. (2)}$$

Here, \( \alpha_i \) is fraction of Env trimers in a viral population that contain \( i \) WT protomers (0 to 3) and \( f \) is the abundance of the WT protomer relative to the total protomer level. As the mutation does not alter Env expression level, \( f \) was equated with the fraction of WT gp160 DNA (relative to total gp160 DNA) used for transfection into virus producing cells. Values for \( \alpha_0, \alpha_1, \alpha_2 \) and \( \alpha_3 \) for each ratio of WT:S375W DNA tested are given in Figure 7.

Dose-response curves of infection inhibition and viral lysis for the different viral populations are shown in Figure 8A, 8B, 8C, and 8D, and the IC50 values are given in Table 4. As expected, both AuNP-KR13 and KR13 were most potent against the 100% WT Env HIV-1 population (4:0) and demonstrated no antiviral activity against the 100% S375W Env HIV-1 population (0:4). For inhibition of viral infection, there was a similar loss of both AuNP-KR13 and KR13 potency as the fraction of WT Env decreased. However, even at the lowest WT Env fraction tested (\( f = 0.125 \) for the 0.5:3.5 population), infection was almost fully blocked at high inhibitor concentrations. In this viral population, the amount of Env trimers with two or three WT protomers (W3 and W2M) is small (~4%), and viruses lacking such Env trimers (i.e., containing only WM2 and M3 trimers) should form a nontrivial fraction of the population. The fact that all viruses of the 0.5:3.5 WT DNA:Mutant DNA population appear sensitive to AuNP-KR13 and KR13 suggests that these inhibitors can effectively block the function of Env trimers containing only one susceptible gp120.

The logarithm of the IC50 values for infection inhibition varied linearly with the fraction of WT protomer in the viral populations (Figure 8E). This linearity suggests that inhibitory potency directly depends on the energy of inhibitor binding. Suppose the energy of dissociation for a single KR13 is \( \Delta G \), then the energy of \( n \) such events is \( n\Delta G \). The average value of this occupancy number \( n \) can be reasonably assumed to vary linearly with the average number of the available sites on HIV-1,
which, in turn, is proportional to $f$. In this simplistic model, effective inhibitory concentrations can be treated as *de facto* equilibrium binding constants, leading to the observed proportionality:

$$\ln(I_{C_{50}}) \propto -f \Delta G$$  \hfill (3)

The linear relationships for AuNP-KR13 and KR13 share the same slope, as expected since single inhibitor-binding energy $\Delta G$ is the same for both species. The result suggests that the two inhibitors utilize the same mechanism to block viral infection and that the enhanced inhibitory activity of AuNP-KR13 is a consequence of its ability to concentrate additional KR13 near the viral surface after the first peptide triazole is bound.

While similar dependence on $f$ was observed for viral lysis induced by AuNP-KR13, the lytic activity of KR13 was markedly different (Figures 8B and 8D). These KR13 titrations were largely independent of the WT protomer fraction until $f$ dropped to 0.25. The KR13 titrations for $f = 0.25$ (1:3) and $f = 0.125$ (0.5:3.5) were significantly shallower than those for $f > 0.25$ and for all of the viral lysis titrations of AuNP-KR13. The divergence in lytic activity between AuNP-KR13 and KR13 is reflected in the different slopes of the $\ln(E_{C_{50}})$ versus $f$ plots (Figure 8F). The AuNP-KR13 data had the same slope as the plots for infection inhibition (Figure 8E), suggesting a similar dependence of lytic activity on KR13 binding energy. By contrast, the KR13 data for $f > 0.25$ had zero slope, implying a fundamental difference in how AuNP-KR13 and KR13 encounter the virus to cause lysis.

**Morphological analysis of AuNP-KR13 conjugates effects on HIV-1**

We sought to image the virus-nanoparticle interface and consequent virus lysis using transmission electron microscopy. Viruses were treated with the AuNP-KR13 (20 nm and 80 nm) conjugates for 10 minutes, and then fixed and loaded on a TEM grid. Uranyl acetate was used as a contrast agent to stain the lipids in order to visualize the virion. Representative images obtained are shown in Figure 9. In the images for both the 20 nm and 80 nm AuNP-KR13, a limited number of AuNP-KR13 particles (average 1-3) were found associated with individual virions, suggesting that a small number of NP’s is sufficient to induce virus lysis. The 80 nm AuNP-KR13 appeared to cause a more drastic disruption of the virion compared to the 20 nm AuNP-KR13. As shown by the representative images in Figure 9, we observed that AuNP-KR13 led to a drastic disruption and collapse of the virus particle, with the residual virion partially engulfing the nanoparticle conjugate. In contrast, KR13 was shown previously (37) to lead to a more subtle virion collapse and at the same time greater virion shrinkage as observed using TEM.

**DISCUSSION**

The current study has demonstrated the ability to dramatically enhance the potency of anti-HIV-1 function of PT inhibitors by gold nanoparticle display, and at the same time has derived a conceptual model of nanoparticle-virus encounter that can hijack the intrinsic metastability of the virus spike protein complex (15, 54-56). Previously, we demonstrated that conjugating PT thiols on 20 nm AuNP improved their anti-viral functions (38). Here, we found that altering the AuNP properties of core size and PT density coverage led to dramatic enhancement in potency of both viral infection inhibition and cell-free virolysis (Figures 2, 3 and 4). More importantly, potency enhancement was also observed in replication competent HIV-1 (Figure 5). The potent antiviral functions of nanoparticle-PT conjugates, in particular irreversible inactivation of viruses by specific interaction with the virus surface protein gp120, makes this a worthy type of inhibition to understand mechanistically.

Considering that both KR13 and AuNP-KR13 exhibit a similar pattern of antiviral activities though with vastly different potencies, we asked if there are fundamental mechanistic differences for these two inhibitor compositions. Observations made in this study (Figure 6) demonstrated important differences. The MPER of gp41 on the HIV-1 Env is known to be exposed during the HIV-1 / host cell fusion process, but to be unexposed in the ground (unliganded) state of the virus (57). For KR13, we previously observed both concentration dependent and time dependent exposure of the MPER epitope on the HIV-1 virion when viruses were treated with KR13 (37). However, in the case
of AuNP-KR13, we did not observe MPER epitope exposure (Figure 6A). In addition, the data in Figure 6B show that T20, a fusion inhibitor that targets 6-helix bundle formation of gp41 during HIV-1 fusion with host cell, inhibits KR13-induced p24 release (as reported previously (37)) but does not inhibit such release by AuNP-KR13 (Figure 6B). Further, the data of Figure 6C show that gp120 shedding, infection inhibition and virolysis tracked by p24-release of the HIV-1 BaL pseudotype all had a similar time-dependence for the AuNP-KR13 exposure, in contrast to the unequal kinetics for KR13 (p24 release lagged compared to gp120 shedding and infection inhibition (37)). We note that the difference in kinetics of virus breakdown could have an impact on the extent of T20 sensitivity detected with AuNP-KR13 treatment. However, the lack of exposure of 4E10 and 2F5 epitopes with AuNP-KR13, vs exposure with KR13, argues that kinetics alone cannot explain the differences seen between the modes of action of these two types of virus inactivators. Collectively, the characteristics of MPER exposure, T20 sensitivity and time-dependence of antiviral effects observed for KR13 all argue that KR13-induced lysis bears a relationship to the physiological events of virus-cell fusion. In contrast, the lack of these phenotypes with AuNP-KR13 suggests that the latter responds to different forces. Several other differences between the mechanism of action of KR13 and AuNP-KR13 have emerged during ongoing investigations of KR13-induced lysis and its relationship to HIV-1 biological functions (58). A gp120 antibody, 2G12, that binds to the V3 loop and inhibits KR13 induced virolysis, has been observed (58) not to inhibit AuNP-KR13 induced virolysis. In addition, DTNB (dithionitrobenzene), a thiol-blocking agent that inhibits KR13 induced virolysis, did not affect AuNP-KR13 mediated virolysis (unpublished results). These data clearly reinforce the need to consider mechanisms specific to gold nanoparticles in order to explain the virolytic activity of AuNP-KR13.

The responses of both p24 release and infection inhibition potency enhancements to nanoparticle and virus surface properties argue that multivalent contact between virus and nanoparticle surfaces is a major driving force for the enhanced antiviral potencies of AuNP-KR13. Three lines of evidence obtained in this work support the conclusion of multivalency. [1] Increasing the size of the AuNP particle leads to dramatic potency enhancement (Figure 2, Table 1). Since size increase causes a decrease of nanoparticle curvature, the area of contact of virus with nanoparticle would be expected to increase, and more points of contact would ensue between the peptide triazole NP surface and multiple spikes on the virus surface. [2] Increased density of KR13 on the AuNP surface strongly enhances anti-viral potency (Figure 3 and Tables 2 and 3). Increased areal peptide density is expected to increase the extent of multipoint attachment between peptide triazoles and Env spikes on the nanoparticle and virus surfaces, respectively. [3] Decreasing the amount of wild-type (fully active) spikes on the HIV-1 virion surface by replacing the gp120 components with the KR13-insensitive mutant gp120, S375W, suppressed KR13-induced p24 release more drastically than AuNP-KR13 activity (Figure 8C and 8D). The functional data (Figure 8) can be correlated with the statistically predicted relative abundance of heterogeneous and homogenous spikes expected for different mutant-WT mixtures (Figure 7). We observed that, while KR13 induced p24 release required a statistical average of ≥1 fully active spike (containing 3 active WT gp120 monomers in individual spikes per virion), AuNP-KR13 induced up to 50% p24 release even with an average of close to zero fully active spikes (the case of 0.5-3.5 WT-mutant). The contrasting effects of KR13 vs AuNP-KR13 induced p24 release (Figure 8F) suggest that AuNP-KR13 can cause lysis by engaging multiple spikes with an average of only 1 active gp120/spike, while KR13 likely requires a greater number of active gp120 monomers per spike trimer. It is worth noting that the ability of both KR13 and AuNP-KR13 to fully inhibit virus at spike populations containing a significant (though not complete) proportion of S375W mutant shows that mixed trimers can still exhibit PT sensitivity to the extent that they contain WT gp120 protomers. The difference in HIV-1 virion encounter by KR13 and AuNP-KR13 is shown schematically in Figure 10. The physical nature of encounter in the model is based on the previously known dimensions of the virus Env (17, 55) and assumes that KR13 components on the AuNP are isotropic and equidistant from each other.
One may ask why multivalent contact should cause such a profound effect leading to irreversible inactivation. We speculate that multivalent contact as envisioned in Figure 10 causes an elevated stress on the HIV-1 membrane of the metastable HIV-1 virion, leading to irreversible collapse of the virion particle. As envisioned in the model in Figure 10, KR13 action requires an Env spike with full or close to full native Env protomer content to exert a lytic force on the virus. In contrast, AuNP-KR13 exerts a lytic force by crosslinking Env spikes and hence can carry out this function as long as multiple Env spikes each contain at least one active protomer.

That AuNP-KR13 causes significant loss in virus integrity was visualized by TEM imaging (Figure 9). Here, the physical impact on the virion is much greater for AuNP-KR13 as compared to KR13 alone (37). Further investigation is needed to better understand the nature of the virus membrane transformation caused by AuNP-KR13 and how this type of effect compares to membrane transformations triggered by other types of virus-lysing agents (59-61).

The work presented here reinforces the potential usefulness of inhibitor display on nanoparticles to improve potency for disease prevention and intervention. While nanocarriers have been used as vehicles for drug delivery, their use for forming therapeutic agents has been more limited (62-65). Jiang et al. have shown that gold nanoparticles ranging from 2-100 nm in diameter conjugated to multiple trastuzumab antibodies enable targeting and cross-linking of human epidermal growth factor receptor (HER)-2 in human SK-BR-3 breast cancer cells, by causing reduced (HER)-2 receptor expression and inducing receptor mediated endocytosis (66). Jiang also showed that the anticancer potencies were altered by variations of the diameter of the gold nanoparticles, with the larger particles having more protein on their surface and increased avidity, while the smaller particles exhibited greater receptor mediated endocytosis (66). In the area of HIV-1, nanomaterial design for HIV-1 infection inhibition has been limited (67). Gold nanoparticle display was found to increase the antiviral potency of the HIV-1 inhibitor SDC-1721, a derivative of TAK-779, that targets the cellular coreceptor, CCR5 (68, 69). However, such studies have used very small (2 nm) AuNP that could be taken up by cells non-specifically (70). Heterogeneity of the HIV-1 also has limited antibody and drug targeting (71). The current work argues that gold nanoparticle display could help overcome the heterogeneity of Env spikes by enabling multivalent attachment of neighboring partially defective spikes. The current work provides a generalizable approach for nanoparticle drug design not only for HIV-1 but also for other infectious enveloped viruses such as influenza and hepatitis B virus.

Several types of inhibitors that bind to the HIV-1 envelope protein gp120 have now been found to be lytic inactivators of HIV-1. These include DAVEI (dual acting virus entry inhibitor containing cyanovirin-N fused to MPER) (61), the dodecameric CD4 construct (72), KR13 and other PT thiols (37, 38, 58) and PT thiols on AuNP’s. There are differences in the modes of action of these inactivators at the molecular level. DAVEI causes lysis but no observed gp120 shedding, and the requirement for the MPER domain for DAVEI lysis argues for unique interactions of this sequence with gp41 and/or membrane of the virus envelope (61). KR13 and AuNP-KR13 both cause gp120 shedding and membrane lysis but differ mechanistically as enumerated above. Nonetheless, in spite of these differences, the common feature of cell-independent virus lysis hints that there likely is a common mechanistic underpinning for all of these inhibitors. We posit that this lies in the intrinsic metastability that has been argued to be a property of HIV-1 Env (15, 54-56) and of the HIV-1 virion itself (73, 74). Metastability is a feature common to all viruses, since these must protect their genetic contents and at the same time be able to open up to transfer their contents during cell entry to propagate more virus (75-77). For HIV-1, we propose that virus metastability appears to be a general property that can be hijacked for specific antiviral activities, as observed previously (37, 38, 61, 72). At the same time, the actions of lytic inactivators can inform about fundamental properties of virus metastability, including the membrane transformations that underlie both virolysis by gp120-inhibitor interactions and virus-cell fusion by gp120-cell receptor interactions.
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REFERENCES

1. Doyle, T., and Geretti, A. M. (2012) Low-level viraemia on HAART: significance and management, *Current opinion in infectious diseases* 25, 17-25.

2. (UNAIDS), J. U. N. P. o. H. A. (2013) Global Report: UNAIDS report on the global AIDS epidemic 2013, In p.v., *Joint United Nations Programme on HIV/AIDS*, p 198.

3. Kromdijk, W., Huitema, A. D., and Mulder, J. W. (2010) Treatment of HIV infection with the CCR5 antagonist maraviroc, *Expert opinion on pharmacotherapy* 11, 1215-1223.

4. Flores, A., and Quesada, E. (2013) Entry inhibitors directed towards glycoprotein gp120: an overview on a promising target for HIV-1 therapy, *Current medicinal chemistry* 20, 751-771.

5. Palmisano, L., and Vella, S. (2011) A brief history of antiretroviral therapy of HIV infection: success and challenges, *Annali dell'Istituto superiore di sanita* 47, 44-48.

6. Granich, R., Crowley, S., Vitoria, M., Lo, Y. R., Souteyrand, Y., Dye, C., Gilks, C., Guerma, T., De Cock, K. M., and Williams, B. (2010) Highly active antiretroviral treatment for the prevention of HIV transmission, *Journal of the International AIDS Society* 13, 1.

7. Haqqani, A. A., and Tilton, J. C. (2013) Entry inhibitors and their use in the treatment of HIV-1 infection, *Antiviral research* 98, 158-170.

8. Qiu, S., Yi, H., Hu, J., Cao, Z., Wu, Y., and Li, W. (2012) The binding mode of fusion inhibitor T20 onto HIV-1 gp41 and relevant T20-resistant mechanisms explored by computational study, *Current HIV research* 10, 182-194.

9. Henrich, T. J., and Kuritzkes, D. R. (2013) HIV-1 entry inhibitors: recent development and clinical use, *Current opinion in virology* 3, 51-57.

10. Wallace, B. J., Tan, K. B., Pett, S. L., Cooper, D. A., Kossard, S., and Whitfeld, M. J. (2011) Enfuvirtide injection site reactions: a clinical and histopathological appraisal, *The Australasian journal of dermatology* 52, 19-26.

11. Perry, C. M. (2010) Maraviroc: a review of its use in the management of CCR5-tropic HIV-1 infection, *Drugs* 70, 1189-1213.

12. Pan, C., Liu, S., and Jiang, S. (2010) HIV-1 gp41 fusion intermediate: a target for HIV therapeutics, *Journal of the Formosan Medical Association = Taiwan yi zhi* 109, 94-105.

13. Champagne, K., Shishido, A., and Root, M. J. (2009) Interactions of HIV-1 inhibitory peptide T20 with the gp41 N-HR coiled coil, *The Journal of biological chemistry* 284, 3619-3627.

14. Emmelkamp, J. M., and Rockstroh, J. K. (2007) CCR5 antagonists: comparison of efficacy, side effects, pharmacokinetics and interactions--review of the literature, *European journal of medical research* 12, 409-417.

15. Chan, D. C., and Kim, P. S. (1998) HIV entry and its inhibition, *Cell* 93, 681-684.

16. Wyatt, R., Kwong, P. D., Desjardins, E., Sweet, R. W., Robinson, J., Hendrickson, W. A., and Sodroski, J. G. (1998) The antigenic structure of the HIV gp120 envelope glycoprotein, *Nature* 393, 705-711.

17. Kwong, P. D., Wyatt, R., Robinson, J., Sweet, R. W., Sodroski, J., and Hendrickson, W. A. (1998) Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody, *Nature* 393, 648-659.

18. Wyatt, R., and Sodroski, J. (1998) The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens, *Science* 280, 1884-1888.

19. Hermann, F. G., Egerer, L., Brauer, F., Gerum, C., Schwalbe, H., Dietrich, U., and von Laer, D. (2009) Mutations in gp120 contribute to the resistance of human immunodeficiency virus type 1 to membrane-anchored C-peptide maC46, *Journal of virology* 83, 4844-4853.

20. Park, E. J., Vujcic, L. K., Anand, R., Theodore, T. S., and Quinnan, G. V., Jr. (1998) Mutations in both gp120 and gp41 are responsible for the broad neutralization resistance of variant human
immunodeficiency virus type 1 MN to antibodies directed at V3 and non-V3 epitopes, *Journal of virology* 72, 7099-7107.

21. Johnson, V. A., Calvez, V., Gunthard, H. F., Paredes, R., Pillay, D., Shafer, R. W., Wensing, A. M., and Richman, D. D. (2013) Update of the drug resistance mutations in HIV-1: March 2013, *Topics in antiviral medicine* 21, 6-14.

22. Madani, N., Princiotto, A. M., Schon, A., Lalonde, J., Feng, Y., Freire, E., Park, J., Courter, J. R., Jones, D. M., Robinson, J., Liao, H. X., Moody, M. A., Permar, S., Haynes, B., Smith, A. B., 3rd, Wyatt, R., and Sodroski, J. (2014) CD4-mimetic Small Molecules Sensitize Human Immunodeficiency Virus (HIV-1) to Vaccine-elicited Antibodies, *Journal of virology*.

23. Nettles, R. E., Schurmann, D., Zhu, L., Stonier, M., Huang, S. P., Chang, I., Krystal, M., Wind-Rotolo, M., Ray, N., Hanna, G. J., Bertz, R., and Grasela, D. (2012) Pharmacodynamics, safety, and pharmacokinetics of BMS-663068, an oral HIV-1 attachment inhibitor in HIV-1-infected subjects, *The Journal of infectious diseases* 206, 1002-1011.

24. Zhao, Q., Ma, L., Jiang, S., Lu, H., Liu, S., He, Y., Strick, N., Neamati, N., and Debnath, A. K. (2005) Identification of N-phenyl-N’-(2,2,6,6-tetramethyl-piperidin-4-yl)-oxalamides as a new class of HIV-1 entry inhibitors that prevent gp120 binding to CD4, *Virology* 339, 213-225.

25. Hughes, A., Barber, T., and Nelson, M. (2008) New treatment options for HIV salvage patients: an overview of second generation PIs, NNRTIs, integrase inhibitors and CCR5 antagonists, *The Journal of infection* 57, 1-10.

26. Nichols, W. G., Steel, H. M., Bonny, T., Adkison, K., Curtis, L., Millard, J., Kabeya, K., and Clumeck, N. (2008) Hepatotoxicity observed in clinical trials of aplaviroc (GW873140), *Antimicrobial agents and chemotherapy* 52, 858-865.

27. Nichols, W. K., Mehta, R., Skordos, K., Mace, K., Pfeifer, A. M., Carr, B. A., Minko, T., Burchiel, S. W., and Yost, G. S. (2003) 3-methylindole-induced toxicity to human bronchial epithelial cell lines, *Toxicological sciences : an official journal of the Society of Toxicology* 71, 229-236.

28. Madani, N., Schon, A., Princiotto, A. M., Lalonde, J. M., Courter, J. R., Soeta, T., Ng, D., Wang, L., Brower, E. T., Xiang, S. H., Kwon, Y. D., Huang, C. C., Wyatt, R., Kwong, P. D., Freire, E., Smith, A. B., 3rd, and Sodroski, J. (2008) Small-molecule CD4 mimics interact with a highly conserved pocket on HIV-1 gp120, *Structure* 16, 1689-1701.

29. Cocklin, S., Gopi, H., Querido, B., Nimmagadda, M., Kuriakose, S., Cicala, C., Ajith, S., Baxter, S., Arthos, J., Martin-Garcia, J., and Chaiken, I. M. (2007) Broad-spectrum anti-human immunodeficiency virus (HIV) potential of a peptide HIV type 1 entry inhibitor, *Journal of virology* 81, 3645-3648.

30. Emileh, A., Tuzer, F., Yeh, H., Umashankara, M., Moreira, D. R., Lalonde, J. M., Bewley, C. A., Abrams, C. F., and Chaiken, I. M. (2013) A model of peptide triazole entry inhibitor binding to HIV-1 gp120 and the mechanism of bridging sheet disruption, *Biochemistry* 52, 2245-2261.

31. Gopi, H. N., Tirupula, K. C., Baxter, S., Ajith, S., and Chaiken, I. M. (2006) Click chemistry on azidoproline: high-affinity dual antagonist for HIV-1 envelope glycoprotein gp120, *ChemMedChem* 1, 54-57.

32. McFadden, K., Fletcher, P., Rossi, F., Kantharaju, Umashankara, M., Pirrone, V., Rajagopal, S., Gopi, H., Krebs, F. C., Martin-Garcia, J., Shattuck, R. J., and Chaiken, I. (2012) Antiviral breadth and combination potential of peptide triazole HIV-1 entry inhibitors, *Antimicrobial agents and chemotherapy* 56, 1073-1080.

33. Tuzer, F., Madani, N., Kamanna, K., Zentner, I., LaLonde, J., Holmes, A., Upton, E., Rajagopal, S., McFadden, K., Contarino, M., Sodroski, J., and Chaiken, I. (2013) HIV-1 Env gp120 structural determinants for peptide triazole dual receptor site antagonism, *Proteins* 81, 271-290.
34. Umashankara, M., McFadden, K., Zentner, I., Schon, A., Rajagopal, S., Tuzer, F., Kuriakose, S. A., Contarino, M., Lalonde, J., Freire, E., and Chaiken, I. (2010) The active core in a triazole peptide dual-site antagonist of HIV-1 gp120, *ChemMedChem* 5, 1871-1879.

35. Ferrer, M., and Harrison, S. C. (1999) Peptide ligands to human immunodeficiency virus type 1 gp120 identified from phage display libraries, *Journal of virology* 73, 5795-5802.

36. Biorn, A. C., Cocklin, S., Madani, N., Si, Z., Ivanovic, T., Samanen, J., Van Ryk, D. I., Pantophlet, R., Burton, D. R., Freire, E., Sodroski, J., and Chaiken, I. M. (2004) Mode of action for linear peptide inhibitors of HIV-1 gp120 interactions, *Biochemistry* 43, 1928-1938.

37. Bastian, A. R., Contarino, M., Bailey, L. D., Aneja, R., Moreira, D. R., Freedman, K., McFadden, K., Duffy, C., Emileh, A., Leslie, G., Jacobson, J. M., Hoxie, J. A., and Chaiken, I. (2013) Interactions of peptide triazole thiols with Env gp120 induce irreversible breakdown and inactivation of HIV-1 virions, *Retrovirology* 10, 153.

38. Bastian, A. R., Kantharaju, McFadden, K., Duffy, C., Rajagopal, S., Contarino, M. R., Papazoglou, E., and Chaiken, I. (2011) Cell-free HIV-1 virucidal action by modified peptide triazole inhibitors of Env gp120, *ChemMedChem* 6, 1335-1339, 1318.

39. Gopi, H., Cocklin, S., Pirrone, V., McFadden, K., Tuzer, F., Zentner, I., Ajith, S., Baxter, S., Jawanda, N., Krebs, F. C., and Chaiken, I. M. (2009) Introducing metallocene into a triazole peptide conjugate reduces its off-rate and enhances its affinity and antiviral potency for HIV-1 gp120, *J Mol Recognit* 22, 169-174.

40. Frens, G. (1973) Controlled nucleation for the regulation of the particle size in monodisperse gold suspensions, *Nature* 241, 2.

41. Haiss, W., Thanh, N. T., Aveyard, J., and Fernig, D. G. (2007) Determination of size and concentration of gold nanoparticles from UV-vis spectra, *Analytical chemistry* 79, 4215-4221.

42. Xie, H., Tkachenko, A. G., Glomm, W. R., Ryan, J. A., Brenneman, M. K., Papanikolas, J. M., Franzen, S., and Feldheim, D. L. (2003) Critical flocculation concentrations, binding isotherms, and ligand exchange properties of peptide-modified gold nanoparticles studied by UV-visible, fluorescence, and time-correlated single photon counting spectroscopies, *Analytical chemistry* 75, 5797-5805.

43. He, J., Choe, S., Walker, R., Di Marzio, P., Morgan, D. O., and Landau, N. R. (1995) Human immunodeficiency virus type 1 viral protein R (Vpr) arrests cells in the G2 phase of the cell cycle by inhibiting p34cdc2 activity, *Journal of virology* 69, 6705-6711.

44. Connor, R. I., Chen, B. K., Choe, S., and Landau, N. R. (1995) Vpr is required for efficient replication of human immunodeficiency virus type-1 in mononuclear phagocytes, *Virology* 206, 935-944.

45. Xiang, S. H., Kwong, P. D., Gupta, R., Rizzuto, C. D., Casper, D. J., Wyatt, R., Wang, L., Hendrickson, W. A., Doyle, M. L., and Sodroski, J. (2002) Mutagenic stabilization and/or disruption of a CD4-bound state reveals distinct conformations of the human immunodeficiency virus type 1 gp120 envelope glycoprotein, *Journal of virology* 76, 9888-9899.

46. Dey, B., Pancera, M., Svehla, K., Shu, Y., Xiang, S. H., Vainshtein, J., Li, Y., Sodroski, J., Kwong, P. D., Mascola, J. R., and Wyatt, R. (2007) Characterization of human immunodeficiency virus type 1 monomeric and trimeric gp120 glycoproteins stabilized in the CD4-bound state: antigenicity, biophysics, and immunogenicity, *Journal of virology* 81, 5579-5593.

47. Aneja R., R. A., Li H., Sundaram R.V.K, Bailey L., Chaiken I. Functional Determinants of Peptide Triazole Inactivators of HIV-1 Utilize Two Conserved Hydrophobic Cavities in Env gp120. In preparation

48. Chojnacki, J., Staudt, T., Glass, B., Bingen, P., Engelhardt, J., Anders, M., Schneider, J., Muller, B., Hell, S. W., and Krausslich, H. G. (2012) Maturation-dependent HIV-1 surface protein redistribution revealed by fluorescence nanoscopy, *Science* 338, 524-528.
49. Muranyi, W., Malkusch, S., Muller, B., Heilemann, M., and Krausslich, H. G. (2013) Super-resolution microscopy reveals specific recruitment of HIV-1 envelope proteins to viral assembly sites dependent on the envelope C-terminal tail, *PLoS pathogens* **9**, e1003198.

50. Crooks, E. T., Tong, T., Osawa, K., and Binley, J. M. (2011) Enzyme digests eliminate nonfunctional Env from HIV-1 particle surfaces, leaving native Env trimers intact and viral infectivity unaffected, *Journal of virology* **85**, 5825-5839.

51. Summers, M. F., Henderson, L. E., Chance, M. R., Bess, J. W., Jr., South, T. L., Blake, P. R., Sagi, I., Perez-Alvarado, G., Sowder, R. C., 3rd, Hare, D. R., and et al. (1992) Nucleocapsid zinc fingers detected in retroviruses: EXAFS studies of intact viruses and the solution-state structure of the nucleocapsid protein from HIV-1, *Protein science : a publication of the Protein Society* **1**, 563-574.

52. Vogt, V. M., and Simon, M. N. (1999) Mass determination of rous sarcoma virus virions by scanning transmission electron microscopy, *Journal of virology* **73**, 7050-7055.

53. Kim, T., Lee, K., Gong, M. S., and Joo, S. W. (2005) Control of gold nanoparticle aggregates by manipulation of interparticle interaction, *Langmuir : the ACS journal of surfaces and colloids* **21**, 9524-9528.

54. Mao, Y., Wang, L., Gu, C., Herschhorn, A., Desormeaux, A., Finzi, A., Xiang, S. H., and Sodroski, J. G. (2013) Molecular architecture of the uncleaved HIV-1 envelope glycoprotein trimer, *Proceedings of the National Academy of Sciences of the United States of America* **110**, 12438-12443.

55. Myszka, D. G., Sweet, R. W., Hensley, P., Brigham-Burke, M., Kwong, P. D., Hendrickson, W. A., Wyatt, R., Sodroski, J., and Doyle, M. L. (2000) Energetics of the HIV gp120-CD4 binding reaction, *Proceedings of the National Academy of Sciences of the United States of America* **97**, 9026-9031.

56. Dimitrov, A. S., Jacobs, A., Finnegan, C. M., Stiegler, G., Katinger, H., and Blumenthal, R. (2007) Exposure of the membrane-proximal external region of HIV-1 gp41 in the course of HIV-1 envelope glycoprotein-mediated fusion, *Biochemistry* **46**, 1398-1401.

57. Lauren D. Bailey, H. L., Caitlin Duffy, Rachna Aneja, Arangassery R. Bastian, Ramalingam V.K. Sundaram, Andrew Holmes, Adel Ahmed, Irwin Chaiken. Spatial Relationship of Virolytic Peptide Triazole Thiol Inhibitors of HIV-1, *In preparation*.

58. Xu, Y., Zhang, C., Jia, L., Wen, C., Liu, H., Wang, Y., Sun, Y., Huang, L., Zhou, Y., and Song, H. (2009) A novel approach to inhibit HIV-1 infection and enhance lysis of HIV by a targeted activator of complement, *Virology journal* **6**, 123.

59. Nakamura, M., Terada, M., Sasaki, H., Kamada, M., and Ohno, T. (2000) Virolysis and in vitro neutralization of HIV-1 by humanized monoclonal antibody hNM-01, *Hygromida 19*, 427-434.

60. Contarino, M., Bastian, A. R., Kalyana Sundaram, R. V., McFadden, K., Duffy, C., Gangupomu, V., Baker, M., Abrams, C., and Chaiken, I. (2013) Chimeric Cyanovirin-MPER recombinantly engineered proteins cause cell-free virolysis of HIV-1, *Antimicrobial agents and chemotherapy* **57**, 4743-4750.

61. Farokhzad, O. C., and Langer, R. (2009) Impact of nanotechnology on drug delivery, *ACS nano* **3**, 16-20.

62. Zhang, L., Gu, F. X., Chan, J. M., Wang, A. Z., Langer, R. S., and Farokhzad, O. C. (2008) Nanoparticles in medicine: therapeutic applications and developments, *Clinical pharmacology and therapeutics* **83**, 761-769.

63. Hamidi, M., Azadi, A., Rafiee, P., and Ashrafi, H. (2013) A pharmacokinetic overview of nanotechnology-based drug delivery systems: an ADME-oriented approach, *Critical reviews in therapeutic drug carrier systems* **30**, 435-467.
65. Heidel, J. D., and Davis, M. E. (2011) Clinical developments in nanotechnology for cancer therapy, *Pharmaceutical research* 28, 187-199.

66. Jiang, W., Kim, B. Y., Rutka, J. T., and Chan, W. C. (2008) Nanoparticle-mediated cellular response is size-dependent, *Nature nanotechnology* 3, 145-150.

67. Mamo, T., Moseman, E. A., Kolishetti, N., Salvador-Morales, C., Shi, J., Kuritzkes, D. R., Langer, R., von Andrian, U., and Farokhzad, O. C. (2010) Emerging nanotechnology approaches for HIV/AIDS treatment and prevention, *Nanomedicine (Lond)* 5, 269-285.

68. Bowman, M. C., Ballard, T. E., Ackerson, C. J., Feldheim, D. L., Margolis, D. M., and Melander, C. (2008) Inhibition of HIV fusion with multivalent gold nanoparticles, *J Am Chem Soc* 130, 6896-6897.

69. Mahmoud, K. A., and Luong, J. H. (2008) Impedance method for detecting HIV-1 protease and screening for its inhibitors using ferrocene-peptide conjugate/Au nanoparticle/single-walled carbon nanotube modified electrode, *Analytical chemistry* 80, 7056-7062.

70. Chithrani, B. D., Ghazani, A. A., and Chan, W. C. (2006) Determining the size and shape dependence of gold nanoparticle uptake into mammalian cells, *Nano letters* 6, 662-668.

71. Poignard, P., Moulard, M., Golez, E., Vivona, V., Franti, M., Venturini, S., Wang, M., Parren, P. W., and Burton, D. R. (2003) Heterogeneity of envelope molecules expressed on primary human immunodeficiency virus type 1 particles as probed by the binding of neutralizing and nonneutralizing antibodies, *Journal of virology* 77, 353-365.

72. Bennett, A., Liu, J., Van Ryk, D., Bliss, D., Arthos, J., Henderson, R. M., and Subramaniam, S. (2007) Cryoelectron tomographic analysis of an HIV-neutralizing protein and its complex with native viral gp120, *The Journal of biological chemistry* 282, 27754-27759.

73. Flint, S. J. (2004) *Principles of Virology: Molecular Biology, Pathogenesis, and Control of Animal Viruses*, ASM Press.

74. Kol, N., Shi, Y., Tsvitov, M., Barlam, D., Shneck, R. Z., Kay, M. S., and Rousso, I. (2007) A stiffness switch in human immunodeficiency virus, *Biophysical journal* 92, 1777-1783.

75. Wallin, M., Ekstrom, M., and Garoff, H. (2005) The fusion-controlling disulfide bond isomerase in retrovirus Env is triggered by protein destabilization, *Journal of virology* 79, 1678-1685.

76. White, J. M., Delos, S. E., Brecher, M., and Schornberg, K. (2008) Structures and mechanisms of viral membrane fusion proteins: multiple variations on a common theme, *Critical reviews in biochemistry and molecular biology* 43, 189-219.

77. Flint, S. J., Enquist, L. W., and Racaniello, V. R. (2008) *Principles of Virology 2 Volume Set*, ASM Press.
FOOTNOTES:

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2- The abbreviations used are: HIV, human immunodeficiency virus; AuNP, Gold nanoparticle; PT, peptide triazole; AuNP-PT, Gold nanoparticle-peptide triazole conjugate; HOS.T4.R5, Human osteosarcoma cell line with stable expression of CD4 and CCR5; p24, viral capsid protein; VLP, virus-like particles(s); gp120, viral envelope protein - glycoprotein 120; gp41, viral envelope protein – glycoprotein 41; MPER, membrane proximal external region; DLS, dynamic light scattering; TEM, transmission electron microscope; AIDS, acquired immunodeficiency syndrome; 293T, human embryonic kidney cell line; FBS, fetal bovine serum; ELISA, enzyme linked immunosorbent assay; OPD, O-phenylenediamine; WT, wildtype; BSPP, Bis (p-sulfonatophenyl) phenylphosphine dehydrate dipotassium salt; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HRP, horse radish peroxidase.
FIGURES LEGENDS:

**Figure 1:** Characterization of the AuNP-KR13 conjugates. (A) Comparison of the theoretical and observed AuNP diameters as a function of citric acid concentration during AuNP synthesis. The diameters of the AuNP that were synthesized (experimental) were measured using dynamic light scattering (DLS) (n=25). (B) The % intensity of the different diameters of the AuNP-KR13 conjugates as measured on the zetasizer using the dynamic light scattering methodology (n=3). (C) Representative TEM images of the AuNP-KR13 conjugates with diameters 13.3 nm, 19.9 nm, 42.8 nm and 92.6 nm. Error bars represent the standard deviation of the mean, n > 3.

**Figure 2:** Size dependent anti-viral effects of the AuNP-KR13 conjugates on the HIV-1 BaL pseudovirions. (A) Inhibition of cell infection measured using a single round infection assay. The IC50 values are reported in Table 1. (B) Relative p24 release from the cell-free HIV-1 BaL pseudovirions with increasing concentration of the different diameter AuNP-KR13 conjugates. The EC50 values are reported in Table 1. Error bars represent the standard deviation of the mean, n = 3.

**Figure 3:** Anti-viral effects of AuNP-KR13 conjugates with increasing diameter of the AuNP with equal density of KR13 coverage per square nm on the surface of the AuNP. (A) Inhibition of HIV-1 infection by the AuNP-KR13 conjugates measured using a single round infection assay as explained in methods. The IC50 values are reported in Table 3. (B) Relative p24 release from the cell-free HIV-1 BaL pseudovirions with increasing concentration of the different diameter AuNP-KR13 conjugates. The EC50 values are reported in Table 3. Error bars represent the standard deviation of the mean, n = 3.

**Figure 4:** Anti-viral effects of AuNP-KR13 (19.9 nm diameter) with increasing surface density coverage of KR13, with 100% being 69 peptides per AuNP as represented in Table 3. (A) Inhibition of cell infection measured using a single round infection assay as explained in methods. The IC50 values are reported in Table 2. (B) Relative p24 release from the cell-free HIV-1 BaL pseudovirions with increasing concentration of the AuNP-KR13 conjugates with decreasing KR13 density coverage. The EC50 values are reported in Table 2. Error bars represent the standard deviation of the mean, n = 3.

**Figure 5:** Characterization of AuNP-KR13 (20 nm and 80 nm) induced infection inhibition and virus breakdown of replication competent HIV-1 BaL virus. (A) Inhibition of cell infection measured using a p24 ELISA of the produced virions in the presence of the inhibitor. The IC50 values of AuNP:KR13 (20 nm) and AuNP:KR13 (80 nm) to inhibit HIV-1 BaL infection were 3.16 nM and 0.73 nM respectively. (B) Relative p24 release from the cell-free HIV-1 BaL (replication competent) with increasing concentration of the 20 nm and 80 nm diameter AuNP-KR13 conjugates. The EC50 values of AuNP:KR13 (20 nm) and AuNP:KR13 (80 nm) to inhibit HIV-1 BaL infection were 8.19 nM and 0.49 nM respectively. Error bars represent the standard deviation of the mean, n = 3.

**Figure 6:** Comparison of the anti-viral effects of AuNP-KR13 and KR13 on the HIV-1 BaL pseudotyped virus. (A) MPER (membrane proximal external region) specific gp41 epitope exposure on viruses treated with AuNP-KR13 and KR13 as measured with MPER specific antibody (2F5 and 4E10) binding to HIV-1 BaL pseudotyped virion after 30 minutes of AuNP-KR13 (20 nm diameter) and KR13 respectively. (B) T20 (Fusion Inhibitor) inhibition of AuNP-KR13 (50 nM) and KR13 (1 µM) induced p24 release of HIV-1 BaL pseudotyped. The IC50 value for T20 inhibition of KR13-induced p24 release was 15.9 ± 4.9 nM using Origin V.8.1 (Origin Lab). No significant effect was observed on the AuNP-KR13 induced p24 release by
the addition of T20. (C) Time dependent anti-viral effects of AuNP-KR13 (20 nm) on the viral infection inhibition p24 and gp120 release. The % of cell infection retained after peptide treatment is shown on the left y-axes, and the viral protein gp120 and p24 retained in the virus fraction is shown on the right y-axes. All samples were adjusted to the untreated virus as full infection and viral protein retention. Each time point of peptide treatment had a control for normalization. Error bars represent the standard deviation of the mean, n = 3.

**Figure 7:** Representation of WT: mutant (S375W) monomer arrangements on the virus spike with different ratios of the WT: mutant DNA concentrations used for virus production. The % of spikes were calculated using the formula in equation (2) (see Results). The obtained ratio, $\alpha$, was multiplied by 100 to obtain percentage of monomer.

**Figure 8:** Impact of WT/mutant gp120 variation on antiviral functions of AuNP-KR13 (A for infection inhibition, C for p24 release) and KR13 (B for infection inhibition, D for p24 release). Assays used HIV-1 BaL pseudovirions with varying concentrations of incorporated S375W mutant spikes incorporated. Inhibition of HIV-1 infection was measured using a single round infection assay as explained in Methods. The cell-free KR13 and AuNP-KR13 (20 nm) induced p24 release from these virions was measured using the p24 ELISA of the released protein as explained in Methods. The EC$_{50}$ values are reported in Table 4. (E) Linear relationship between the natural log of the IC$_{50}$s of the virus infection inhibition (Table 4) by AuNP-KR13 and KR13, and the fraction of the WT DNA in the HIV-1 virions with linear fits that have R-values > 0.97. (F) Data analysis showing that only the natural log of the EC$_{50}$s of the p24 release, induced by AuNP-KR13 (Table 4), and the fraction of WT DNA in the HIV-1 virion, have a linear relationship with R-value = 0.973, while the KR13 induced p24 release does not follow this pattern. Error bars represent the standard deviation of the mean, n = 3.

**Figure 9:** Representative TEM images for HIV-1 BaL pseudotyped virus treated with PBS (top), 20 nm AuNP (middle) or 80 nm AuNP (bottom). After treatment for 10 minutes at 37°C, samples were fixed as explained in the Methods. Collapsed virions of the type shown were validated to be virus-derived, as opposed to impurities, by detecting distinctive element signatures using the EDAX system attached to the TEM instrument. The samples were stained with 1% uranyl acetate, loaded onto a TEM copper grid and imaged using the JEM 2100 operated at 120 kV (JEOL, Japan).

**Figure 10:** Model comparing the lysis-inducing encounter of KR13 and AuNP-KR13 with HIV-1 virus. The diameters of the HIV-1 virus as well as the AuNP-KR13 conjugates are taken from the measured values by DLS and TEM respectively. The width and height of a spike was taken from the cryo-TEM modeling of Mao et al. 2012 (55).
Table 1: Anti-viral effects of AuNP-KR13 with varying diameter and peptide density on the AuNP surface. The viral infection IC\textsubscript{50} and p24 release EC\textsubscript{50} were obtained from Figure 2 using Origin Pro. 8. The average number of peptides per AuNP were obtained using amino acid analysis. N =3.

| Diameter (nm) | Viral infection inhibition IC\textsubscript{50} (nM) | p24 release EC\textsubscript{50} (nM) | Average number of peptides per AuNP | Peptide density per nm\textsuperscript{2} |
|---------------|---------------------------------|---------------------------------|-----------------------------------|---------------------------------|
| 13.2          | 60 ± 10                          | 10.95 ± 1.66                    | 34                                | 0.062                           |
| 19.9          | 0.9 ±0.2                         | 14.16 ± 1.52                    | 73                                | 0.059                           |
| 42.8          | 0.18 ± 0.1                       | 12.10 ± 0.83                    | 139                               | 0.024                           |
| 92.6          | 0.02 ± 0.01                      | 9.85 ± 0.61                     | 220                               | 0.008                           |
| 123.4nm       | 0.01 ± 0.01                      | 5.71 ± 0.60                     | 652                               | 0.014                           |
**Table 2:** Anti-viral effects of AuNP-KR13 with varying diameter at constant peptide density per square nm on the AuNP surface. The viral infection IC₅₀ and p24 release EC₅₀ were obtained from Figure 3 using Origin Pro. 8. The average number of peptides per AuNP were obtained using amino acid analysis. N =3.

| Inhibitor Used | Viral Infection Inhibition IC₅₀ (nM) | P24 release EC₅₀ (nM) | Coverage density (no. of peptides) | Peptide density per nm² |
|----------------|-------------------------------------|-----------------------|-----------------------------------|------------------------|
| KR13           | 196.6 ± 36.29                       | 3250 ± 567            | 1                                 | 1                      |
| AuNP-KR13 (20.2 nm) | 5.3 ± 1.14                          | 9.7 ± 1.1             | 96                                | 0.075                  |
| AuNP-KR13 (42.3 nm) | 0.7 ± 0.14                           | 1.2 ± 2.5             | 430                               | 0.077                  |
| AuNP-KR13 (80.2 nm) | 0.05 ± 0.06                          | 0.2 ± 0.4             | 1387                              | 0.069                  |
Table 3: Anti-viral effects of AuNP-KR13 with constant diameter (19.9 nm) and varying peptide density per square nm on the AuNP surface. The viral infection IC$_{50}$ and p24 release EC$_{50}$ were obtained from Figure 4 using Origin Pro. 8. The average number of peptides per AuNP were obtained using amino acid analysis. N =3.

| Average no. of KR13 per AuNP | Viral infection inhibition | p24 release | Peptide density per nm$^2$ |
|-----------------------------|---------------------------|------------|--------------------------|
|                             | IC$_{50}$ (nM)            | EC$_{50}$ (nM) |                          |
| 68.6                        | 0.42                      | 1.64       | 0.055                    |
| 28.8                        | 1.1                       | 6.2        | 0.023                    |
| 10.9                        | 8.1                       | 19.5       | 0.009                    |
| 0                           | >50 nM AuNP               | >50 nM AuNP | 0                        |
Table 4: Anti-viral effects of AuNP-KR13 (19.9 nm) and KR13 with HIV-1 pseudovirions expressed with varying WT to mutant (S375W) gp160 DNA ratio. The viral infection IC_{50} and p24 release EC_{50} were obtained from Figure 8 using origin Pro. 8. The relative amounts of DNA of WT vs S375W gp160 used for transfection to produce virions with different ratios of WT and S375W gp120 are listed. N = 3.

|                | AuNP-KR13 |               |               |
|----------------|-----------|---------------|---------------|
| µg of DNA BAL-01 WT-S375W | IC_{50} of Viral Infection Inhibition (nM) | EC_{50} of p24 Release (µM) |
| 0.4-3.5        | 2930 ± 316 | 215.1 ± 89.6  |
| 1-3            | 469.6 ± 67 | 159.9 ± 39.5  |
| 2-2            | 151.4 ± 60 | 22.5 ± 1.6    |
| 3-1            | 40.6 ± 4.3 | 10.2 ± 0.8    |
| 3.5-0.5        | 16.5 ± 2.2 | 1.8 ± 0.3     |
| 4-0            | 10.4 ± 2.6 | 1.3 ± 0.1     |
|                | KR13      |               |               |
| µg of DNA BAL-01 WT-S375W | IC_{50} of Viral Infection Inhibition (nM) | EC_{50} of p24 Release (µM) |
| 0.4-3.5        | 22800 ± 3191 | NA           |
| 1-3            | 1412.8 ± 213.3 | NA          |
| 2-2            | 614.1 ± 89.4  | 4.26 ± 0.32  |
| 3-1            | 243.4 ± 32.1  | 6.03 ± 0.54  |
| 3.5-0.5        | 119.2 ± 45.6  | 6.74 ± 1.31  |
| 4-0            | 53.2 ± 12.8   | 3.10 ± 2.5   |
FIGURES:

Figure 1

A. Graph showing the theoretical and experimental diameters of AuNPs (Frens et al. 1973) against the concentration of citric acid (µM).

B. Histogram showing the % intensity distribution of various AuNP diameters (13.2 nm, 19.9 nm, 42.8 nm, 92.6 nm, 123.4 nm).

C. Images of AuNPs with diameters of 13.3 ± 7 nm, 19.9 ± 6 nm, and 42.8 ± 10 nm.
Figure 2:

A) Graph showing the percentage of viral infection (% Viral Infection) against the concentration of KR13 Equivalent (µM). The graph includes data points for different concentrations: 13.2 nm, 19.9 nm, 42.8 nm, 92.6 nm, and 123.4 nm.

B) Graph showing the percentage of P24 release (% Relative P24 Release) against the concentration of KR13 Equivalent (nM). The graph includes data points for different concentrations: 13.2 nm, 19.9 nm, 42.8 nm, 92.6 nm, and 123.4 nm.
Figure 3:
Figure 4:
Figure 5:
Figure 6:

A) Relative gp41 content (ng/ml) vs. Concentration of KR13 Equivalent (μM)

B) % Relative p24 Release vs. Concentration of T20 (nM)

C) % Viral infection/Viral protein vs. Time (min)

- 2F5 (KR13 Treated Virus)
- 4E10 (KR13 Treated Virus)
- 2F5 (AuNP-KR13 Treated Virus)
- 4E10 (AuNP-KR13 Treated Virus)

- KR13
- AuNP-KR13
Figure 7:

| WT DNA-Mutant DNA (μg) | % of spikes |
|------------------------|-------------|
| 4-0                    | 100 0 0 0   |
| 3.5-0.5                | 67 29 4 0.2|
| 3-1                    | 42 42 14 2 |
| 2-2                    | 12.5 37.5 37.5 12.5 |
| 1-3                    | 2 14 42 42 |
| 0.5-3.5                | 0.2 4 29 67 |
| 0-4                    | 0 0 0 100 |
Figure 8:
Figure 9:
Mechanism of Multivalent Nanoparticle Encounter with HIV-1 for Potency Enhancement of Peptide Triazole Virus Inactivation

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