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Synergistic Inhibition of HIV-1 Replication by a Combination of Viral Inhibitors Isolated from Compounds Targeting Viral Entry, Integration and Proviral Transcription of Clinical Isolates and Drug-resistant Strains

Ibrahim S. Abd-Elazem[1], Kiattisak Lugsanangarm[2], Nadtanet Nunthaboot[3] and Ru Chih C. Huang[4]*

[1]*Department of Otolaryngology-Head and Neck Surgery, Johns Hopkins University, School of Medicine, CRB2, Baltimore, Maryland, 21231, USA*
[2]*Department of Chemistry, Bansomdejchaopraya Rajabhat University, Bangkok 10600, Thailand*
[3]*Department of Chemistry and Center of Excellence for Innovation in Chemistry, Mahasarakham University, Mahasarakham, 44150 Thailand*
[4]*Department of Biology, Johns Hopkins University, Baltimore, Maryland, 21218, USA*

Abstract

Background: Drug resistance associated with HIV-1 variants emerges due to undetected treatment failures. Therefore, it is urgent to search for an antiretroviral therapy to control HIV replication. We examined the synergistic inhibition of HIV-1 replication by a combination of potential inhibitors against clinical isolates and drug-resistant strains.

Methods: Nontoxic, natural product-derived inhibitors, the viral entry inhibitor (Gen-1 (tieghemelin, a triterpenoid saponin)), the integrase inhibitor (M522 (lithospermic acid)), and the transcription inhibitors (G4N (tetraglycylated NDGA) or M4N (tetra-O-methyl-NDGA, terameprocol)) were used to target viral entry, integration, and transcription steps. They have been tested against the replication of an AZT-resistant strain of HIV-1 and clinical isolates from HIV-infected patients, where they were examined alone and, in their combinations, to inhibit HIV replication in human H9 cells and PBMCs. The binding conformations and important interactions of all these inhibitors have been evaluated by molecular docking studies.

Results: The IC50 values for Gen-1, M522, and G4N were 20.0 μM, 2.2 μM, and 14.0 μM, respectively, when tested individually, while in combination, it was 1.2 μM when tested in HIV-1RTMF (AZT-resistant strain) infected PBMCs, whereas 65.0 μM, 18.0 μM, and 27.0 μM individually and in combination, it was 3.0 μM when tested against the clinical isolate. The effectiveness of the three inhibitors in combination was evaluated using the calculation of the combination index, where strong synergy was observed for the three inhibitors across all effect levels. Molecular docking calculations revealed that all inhibitors can bind and form chemical interactions *via* H-bond, π…π interaction, and hydrophobic interaction with the corresponding target.

Conclusion: We concluded that the targeted three-drug combination effectively blocked three steps of the life cycle of HIV-1 and prevented viral entry, integration, and transcription processes of the virus with high efficacy, which exhibited potent synergistic drug activities without any toxicity.

Keywords: HIV-1 • Antiretroviral • Lithospermic acid • Tieghemelin • Terameprocol • Synergy

Introduction

Combination Antiretroviral Therapy (ART) has completely transformed the prognosis of Human Immunodeficiency Virus 1 (HIV-1) infection from a fatal disease to a manageable chronic condition [1]. Current combination ART regimens are highly effective in controlling the viral replication of HIV. Their widespread use has led to a significant reduction in the morbidity and mortality associated with HIV infection, as well as the suppression of opportunistic infections [2-5]. Combinations of antiretroviral drugs are critical to controlling viremia and mortality due to HIV-1 infection [6]. An antiretroviral regimen for a treatment-naive patient generally consists of two Nucleoside Reverse Transcriptase Inhibitors (NRTIs) in combination with a third active drug from one of three drug classes: An integrase strand transfer inhibitor, a non-NRTI, or a protease inhibitor with a pharmacokinetic enhancer [7].

A triple-drug combination consisting of antiretrovirals targeting the virus in two steps in the viral life cycle is the current standard of care for ART [8,9]. Most antiviral drugs are small-molecule viral inhibitors that target different stages of the viral life cycle [10]. For example, anti-HIV drugs inhibit viral infection by targeting viral proteins functioning at different stages of HIV replication, such as surface glycoprotein, reverse transcriptase, integrase, and protease [11]. The doses of the drugs in the combinations can be reduced, and thus their toxicity and cost can be decreased accordingly [12]. These combinations may also increase the resistance barrier, thus delaying the emergence of drug resistance. Some studies have shown that combined ART (two or more drugs from two different classes) is the most effective, with benefits that go beyond clinical management of the disease, playing a crucial role in preventing HIV transmission [13]. The emergence of resistant HIV variants to antiretroviral therapies and long-term drug-associated toxicities highlights a continual need for new therapies and the development of new anti-HIV drugs targeting different steps of the HIV life cycle [14-16].

We previously reported on the isolation of several natural plant products that have anti-HIV-1 activity [17-19]. Saponins are amphiphilic molecules composed of a lipophilic aglycone and one or more hydrophilic sugar moieties that are widely distributed among flowering plants and some marine invertebrates and function in the defense of these organisms [20,21].

***Address for Correspondence:** Ru Chih C. Huang, Department of Biology, Johns Hopkins University, Baltimore, Maryland, 21218, USA, E-mail: rhuang@jhu.edu*

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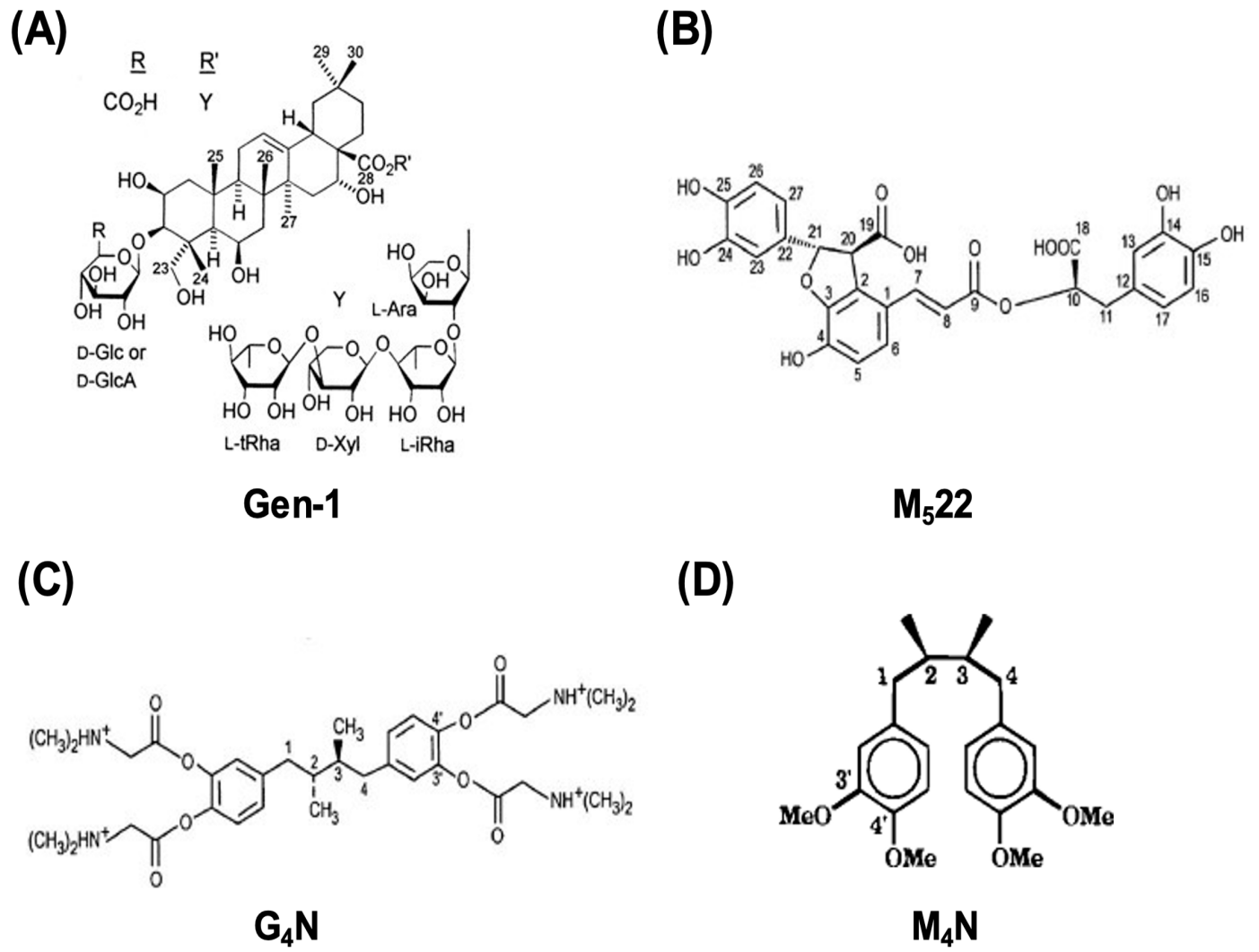

Figure 1. Chemical structures of Gen-1, M522, G4N, and M4N inhibitors. **A)** Viral entry, Gen-1 (tieghemelin), **B)** Integrase inhibitor, M522 (lithospermic acid), **C)** Transcription inhibitors, G4N (tetraglycylated NDGA), and **D)** M4N (tetra-O-methyl-NDGA, terameprocol).

The biological and pharmacological effects of saponins are linked to their effects on cell membranes. Their activities are well documented and include antimicrobial, antifungal, antiviral, anticancer, and immunomodulatory effects [22]. Tieghemelin (Gen-1), a triterpenoid saponin, was isolated from the fruit of the rain forest plant Tieghemella heckelii. Among several saponin congeners, Gen-1 inhibits HIV-1 envelope protein mediated cell-cell fusion at noncytotoxic concentrations with minimal cytotoxicity [19].

Lithospermic acid (M522), a water-soluble metabolite of the Chinese herb danshen (Salvia miltiorrhiza), was shown to inhibit HIV-1 integrase activity, non-toxic to H9 human cells [17] and demonstrate high potency against the replication of various strains of HIV-1, including the clinically resistant strain of the raltegravir-resistant virus [23]. The antitumor, anti-inflammatory and anticoagulant characteristics of Salvia miltiorrhiza and its chemical constituents are well documented [24]. Salvia miltiorrhiza has been widely used in China to improve blood circulation, relieve blood stasis, and treat coronary heart disease. M522, one of the major compounds present in Salvia miltiorrhiza, shares a similar structure with salvianolic acid B, exhibits similar anti-oxidative and cardioprotective activity [25].

Tetra-O-methyl Nordihydroguaiaretic Acid (NDGA) (also known as M4N, terameprocol) is a derivative of a naturally occurring lignan isolated from the creosote bush, Larrea tridentata (D.C.) Coville. It was found to inhibit HIV basal transcription and Tat-regulated transactivation, induce protection of lymphoblastoid cells from HIV killing, and suppress the replication of HIV-1 in stimulated Peripheral Bood Mononuclear Cells (PBMCs). The targets of Mal.4 and M4N are highly conserved Sp1 cognate binding sites in the HIV-1 Long Terminal Repeat (LTR) promoter [18,26]. Because of the challenges associated with the poor water solubility of M4N, a water-soluble derivative, tetraglycyl NDGA (G4N), with similar antiviral activity, was synthesized [27].

In the current study, we evaluated the efficacy of the viral entry, integrase, and proviral transcription inhibitors alone and in a three-drug combination against HIV-1 replication infected cells with drug-resistant strains and HIV-1 clinical isolates from HIV-infected patients. Moreover, we showed the molecular binding modes of these inhibitors with their corresponding targets.

Methods

Inhibitors

The antiviral inhibitors tieghemelin (Gen-1, Figure 1A), M522 (lithospermic acid, Figure 1B), tetraglycyl NDGA (G4N, Figure 1C) and tetra-O-methyl-NDGA (M4N, terameprocol, Figure 1D) were generated as previously described [17,19,26,27]. Stock solutions of the water-insoluble M4N were prepared in a vehicle composed of polyethylene glycol 300 (PEG 300), hydroxypropyl-β-cyclodextrin, and water designated as CPE buffer [28].

Cells and viruses

Human H9 cells and HIV-1RTMF, an AZT-resistant strain of HIV-1, were obtained from the National Institutes of Health (NIH) Acquired

Immunodeficiency Syndrome (AIDS) Reagent Program, Division of AIDS, and National Institute of Allergy and Infectious Diseases. H9 cells were grown and maintained in RPMI 1640 medium supplemented with 10% Fetal Bovine Serum (FBS), 100 units/mL penicillin, and 100 mg/mL streptomycin. Uninfected human blood was collected from a healthy donor, and PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation. These cells were maintained in RPMI 1640 medium supplemented with 15% FBS and stimulated with 2 µg/mL phytohemagglutinin and 32 units/mL human interleukin 2 (IL-2). Virus stocks of the clinical isolates were generated by co-culture of PBMCs from a single healthy donor with lymphocytes from an HIV-infected patient, which were supplied from the Johns Hopkins Biological Repository at the Bloomberg School of Public Health. The toxic effects of the inhibitors on the human H9 T-lymphocyte cells were assessed using the MTT assay as previously described [17].

Viral infection and drug treatment

Details of viral infection for human H9 cells with HIV-1RTMF virus and drug treatment with the different concentrations of inhibitors are previously described [17,23]. Briefly, H9 cells were incubated with the HIV-1RTMF virus at 37 ℃ for 2 h. H9 cells were suspended in culture medium at 1 × 105 cells per mL and infected with HIV at a multiplicity of infection of 0.1. After infection, the cells were washed twice with phosphate-buffered saline, followed by the culture medium. The cell suspension (100 µL) was added to each well of a 96-well plate, and then serial dilutions of each inhibitor alone (Gen-1, M522, and G4N) and in combination were added to the infected cells. After a 4-day incubation, the cells were subcultured with fresh culture medium containing the serial concentrations of the three inhibitors alone and in combination for further incubation until day 8 after infection. The antiviral activities of Gen-1, M522, and G4N in H9 cells infected with HIV-1RTMF (AZT-resistant strain) were determined using the HIV-1 p24 antigen Enzyme-Linked Immunosorbent Assay (ELISA), alone and in combination. Both the infection of the cells with HIV-1 and the p24 antigen ELISA are previously described [17,23].

In another experiment, PBMC cells (5x106 cells/ml) were infected with HIV-1RTMF (AZT-resistant strain) or with a clinical isolate from an HIV-infected patient and then incubated at 37 ℃ for 2 hours. After infection, the cells were washed twice with culture medium (RPMI 1640), suspended with fresh medium, and plated in a 24-well plate. Various concentrations of each drug alone and in combination were added to the cells (0, 1.25, 2.5, 5, 10, 20, 40, 80 µM). After a4-day incubation at 37 ℃, PBMC cells were subcultured with fresh culture medium containing appropriate concentrations of the inhibitors and incubated further until day 8 after infection. By using HIV-1 p24 antigen ELISA, antiviral activities against the drug-resistant strain and the clinical isolate in PBMC were determined. The IC50 values were calculated using dose-response curves for each drug alone, as well as for each combined group (Gen-1, M522, and G4N) and (Gen-1, M522 and M4N) against the replication of HIV drug-resistant strains and clinical isolates from HIV-infected patients. The IC50 ± SD indicated the antiviral activity, where we calculated the Standard Deviation (SD) using KaleidaGraph version 4.0 software.

Drug combination analysis

Analysis of the inhibitor combinations for synergy was performed with CompuSyn software [29] using the Combination Index (CI) isobologram method of Chou and Talalay [30], which is based on the median-effect principle. With this method, additive, synergistic, or antagonistic effects are indicated by CI values of 1, <1, and >1, respectively.

Calculation of docking

The compounds Gen-1 (tieghemelin), G4N (tetraglycyl NDGA), and M4N (tetra-O-methyl-NDGA, terameprocol) were modeled and subjected to geometric optimization at the AM1 level of theory by using the Gaussian 09 program [31]. To study molecular binding of compounds of interest, the receptors were retrieved from the RCSB Protein Data Bank [Research Collaborator for Structural Bioinformatics (http://www.rcsb.org/ pdb)] by PDB code: 1AIK for glycoprotein 41 (gp41) [32] and 1ZJF for transcription factor Sp1 [33]. The Kollman united atom charges have been employed for all receptors, and the Gasteiger-Marsili empirical atomic partial charges [34] have been subsequently employed for all inhibitors. Molecular docking calculations were carried out using the Autodock VINA program [35]. The Gen-1 compound was docked into gp41 protein, and the binding site was defined from the selection of the residues Lys574, Gln577, Leu568, and Trp571 with the grid size of 45 × 45 × 45 points (grid space of 1.0 Å). The G4N and M4N were docked into the Sp1 binding site with a grid size of 30 × 30 × 30, and the other parameters were left at their default settings.

We also carried out more docking calculations for M4N and M522 (lithospermic acid) compounds using DockingServer [36]. The MMFF94 force field [37] was used for energy minimization of ligand molecules M4N and M522 using DockingServer. Docking calculations for M4N were carried out on CTGGGCGGGACT [38], which is present in the Long Terminal Repeats (LTR) region of the HIV-1 genome, and for both M4N and M522 compounds were carried out on AGGGGCGGCGACT [39], which is present in the following LTR region of the HIV-1 genome. Essential hydrogen atoms, Kollman united atom type charges, and solvation parameters were added with the aid of AutoDock tools [40]. Affinity (grid) maps of 30 × 30 × 30 Å grid points and 0.375 Å spacing were generated using the Autogrid program for M4N, while 40 × 40 × 40 Å grid points and 0.375 Å spacing for M522 [40]. The van der Waals and electrostatic terms were calculated using the AutoDock parameter set and distance-dependent dielectric functions, respectively. Docking simulations were performed using the Lamarckian Genetic Algorithm (LGA) and the Solis & Wets local search method [41]. The initial positions, orientations, and torsions of the ligand molecules were set randomly. The supplementary data has the sequence of the LTR and the ⊠-gag region after the LTR region of HIV-1. It shows schematic representations of the HIV-1 genome, essential steps in the HIV life cycle, and targets of ARTs, which include the references used in these data.

Results

Inhibition of the replication of an AZT-resistant strain of HIV-1 in the H9 human T-cell line and in isolated PBMCs by a combination of three viral inhibitors

Inhibitors that target three distinct steps of the HIV-1 life cycle, namely, Gen-1 against viral entry, M522 to prevent integration, and either G4N or M4N to stop proviral transcription in infected cells, were tested for their ability to halt the replication of an AZT-resistant strain of HIV-1 (HIV-1RTMF) in the human

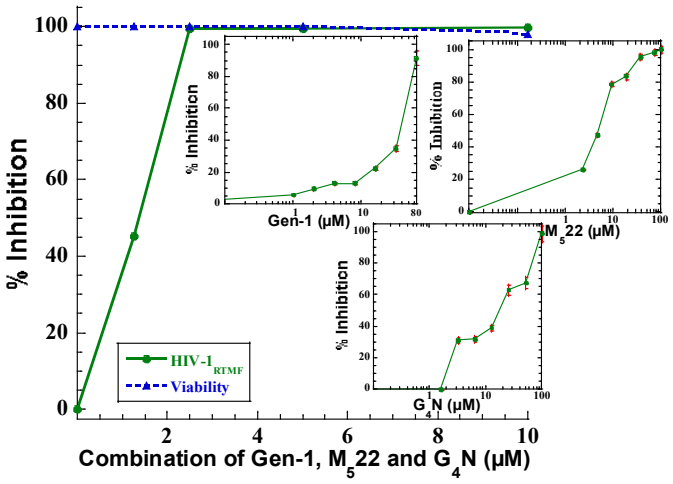

Figure 2. Effect of viral entry, integrase, and transcription inhibitors, alone and in combination, on the replication of AZT-resistant HIV-1 and toxicity of the combined inhibitors on human H9 cells. H9 cells infected with AZT-resistant HIV-1RTMF were treated with serial dilutions of the viral entry inhibitor Gen-1, the integrase inhibitor M522, and the transcription inhibitor G4N, individually and in combination. Human H9 cells were assayed for viral replication, viability, and toxicity effects of the inhibitors on the cells. Dose-response curves were generated from the results of three independent experiments performed in duplicate.

H9 T-lymphocyte cell line and in isolated human PBMC. Inhibitors alone, Gen-1, M522, and G4N were able to inhibit replication of the drug-resistant strain in H9 cells with IC50 values of 40.0 ± 1.20 μM, 4.6 ± 1.38 μM, and 18.0 ± 1.02 μM, whereas the combination of these three inhibitors strongly inhibited the replication of the same drug-resistant strain with an IC50 value of 1.3 ± 1.08 μM without any toxicity of the combined inhibitors on human H9 T-lymphocyte cells as resulted by the viability curve (Figure 2, Table 1). Using the median effect analysis of Chou and Talalay [30], the combination index (CI) for the three inhibitor combinations was determined. The results showed very strong synergism at different effect levels: CI=0.030 at median effective dose (ED50), CI=0.037 at ED75, CI=0.047 at ED90, and CI=0.056 at ED95 (Table 2).

Similar experiments were carried out with HIV-1RTMF -infected PBMCs. As with virus-infected H9 cells, the three viral inhibitors effectively inhibited viral replication of the drug-resistant strain, both alone and with great potency when used in combination (Figure 3A). IC50 values of 20.0 ± 0.42 μM, 2.2 ± 0.83 μM, and 14.0 ± 0.25 μM for Gen-1, M522, and G4N, respectively. In addition, the combination of these three inhibitors strongly inhibited the replication of an AZT-resistant strain with a low IC50 value of 1.2 ± 1.30 μM (Figure 3A, Table 1). When M4N, a water-insoluble, methylated NDGA derivative with antiviral activity [26,42] and approved for clinical trials as an anticancer agent [28,43], was substituted for G4N, similar results were obtained (Figure 3B, Table 1). Therefore, when we combined M4N with these two inhibitors (Gen-1 and M522) against the viral infection of an AZT-resistant strain in PBMC, we found that this combination of drugs highly suppressed the replication of the virus with an IC50 value of 1.0 ± 0.75 μM, especially with the high efficacy of M522 for inhibiting HIV-1 replication (Figure 3B, Table 1). Analysis of the three inhibitor combinations against the drug-resistant strain in PMBCs showed a strong synergistic antiviral effect with CI values of 0.138, 0.112, 0.095, and 0.086 at the ED50, ED75, ED90, and ED95 effect levels, respectively, for the Gen-1, M522, and G4N combinations; and CI values of 0.000199, 0.000639, 0.002, and 0.005, respectively, for the Gen-1, M522, and M4N combinations (Table 2).

Efficacy of the three-drug combination against viral replication of a clinical HIV-1 isolates from an HIV-infected patient

As an initial step towards evaluating the utility of the inhibitor combination to suppress HIV-1 replication in patients with AIDS, the inhibitor regimen was tested with PBMCs infected *in vitro* with clinical isolates of HIV-1 from an HIV-

Table 1. Efficacy of viral entry (Gen-1), integrase ($M_5$22) and transcription inhibitors (G_4N or M_4N) against the AZT-resistant HIV-1_{RTMF} and clinical isolate from an HIV-infected patient, alone and in triple combination in human H9 cells and PBMC.

H9 Cells				PBMC			
Inhibitor(s)	IC_{50}[a]	Inhibitor(s)	IC_{50}	Inhibitor(s)	IC_{50}	Inhibitor(s)	IC_{50}
Alone or Triple	HIV-1_{RTMF}	Alone or Triple	HIV-1_{RTMF}	Alone or Triple	HIV-1_{RTMF}	Alone or Triple	Clinical Isolate
Gen-1	40.0 ± 1.20	Gen-1	20.0 ± 1.42	Gen-1	20.2 ± 0.42	Gen-1	65.0 ± 0.69
$M_5$22	4.6 ± 1.38	$M_5$22	2.2 ± 0.3	$M_5$22	2.2 ± 0.83	$M_5$22	18.0 ± 0.66
G_4N	18.0 ± 1.02	G_4N	14.0 ± 0.25	M_4N	12.0 ± 1.47	G_4N	27.0 ± 1.65
Gen-1=$M_5$22+G_4N	1.3 ± 1.08	Gen-1=$M_5$22+G_4N	1.2 ± 1.30	Gen-1=$M_5$22+M_4N	1.0 ± 0.75	Gen-1=$M_5$22+G_4N	3.0 ± 1.28

Table 2. Combination index values for the three-drug combinations against AZT-resistant HIV-1_{RTMF} and clinical isolates.

Triple Combination of Drug	Virus Infected Cells	CI[b] (combination index) Values at			
		ED^{c}_{50}	ED_{75}	ED_{90}	ED_{95}
Gen-1=$M_5$22+G_4N	H9+HIV-1_{RTMF}	0.03	0.037	0.047	0.056
Gen-1=$M_5$22+G_4N	PBMC+HIV-1_{RTMF}	0.138	0.112	0.095	0.086
Gen-1=$M_5$22+M_4N	PBMC+HIV-1_{RTMF}	0.000199	0.000639	0.002	0.005
Gen-1=$M_5$22+G_4N	PBMC+Clinical Isolate from HIV-infected patient	0.164	0.199	0.258	0.313

[a]IC_{50}, Concentration of inhibitor(s) required to inhibit viral replication by 50% and calculates as (μM) ± SD. Results from three independent experiments performed in duplicate.
[b]CI, Combination index (CI<1, synergism; CI=1, additive effect; CI>1, antagonism).
[c]ED, inhibitor concentration required to achieve the indicated level of inhibition (effect).

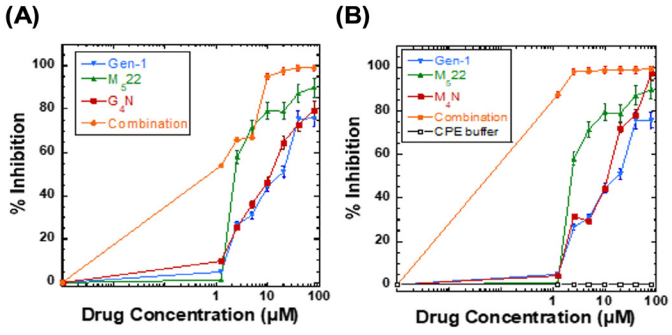

Figure 3. Inhibition of AZT-resistant HIV-1 in PBMCs by viral entry, integrase and transcription inhibitors, alone and in combination. PBMCs were infected with HIV-1RTMF virus and treated with serial concentrations of the viral entry Gen-1, integrase inhibitor M522, and transcription inhibitors, either G4N or M4N. Dose response curves for the inhibition of HIV-1RTMF replication in PBMCs by **A)** Gen-1, M522, and G4N, tested alone and in combination, while **B)** Gen-1, M522, and M4N, tested individually and in combination. The results were determined from three independent experiments performed in duplicate.

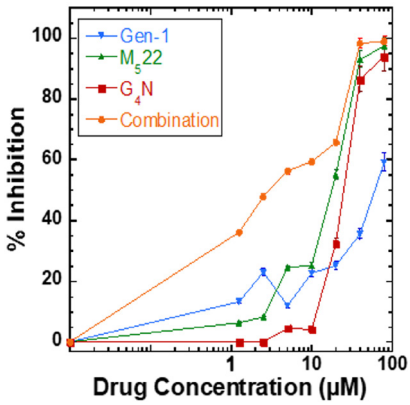

Figure 4. Inhibition of the replication of a clinical isolate of HIV-1 in PBMCs. Dose-response curves for PBMCs infected with a HIV-1 clinical isolates from an HIV-infected patient treated with serial dilutions of Gen-1, M522, and G4N, alone and in combination. HIV replication was determined for three independent experiments performed in duplicate with the HIV-1 p24 antigen ELISA.

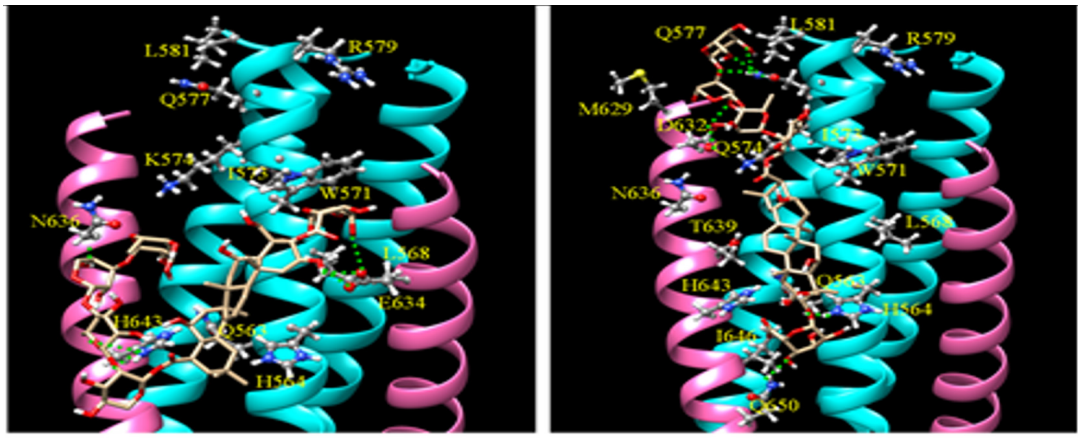

Figure 5. Binding mode of the Gen-1 compound bound to gp41 protein. Two possible modes of action of Gen-1 are shown as bend mode (left panel) and straight mode (right panel). The NHR is shown in cyan and the CHR in pink. All hydrogen bonds are shown as green dashed lines. The figures were prepared using UCSF Chimera software version 1.11.2 [66], (to see this figure in color, refer to the web version).

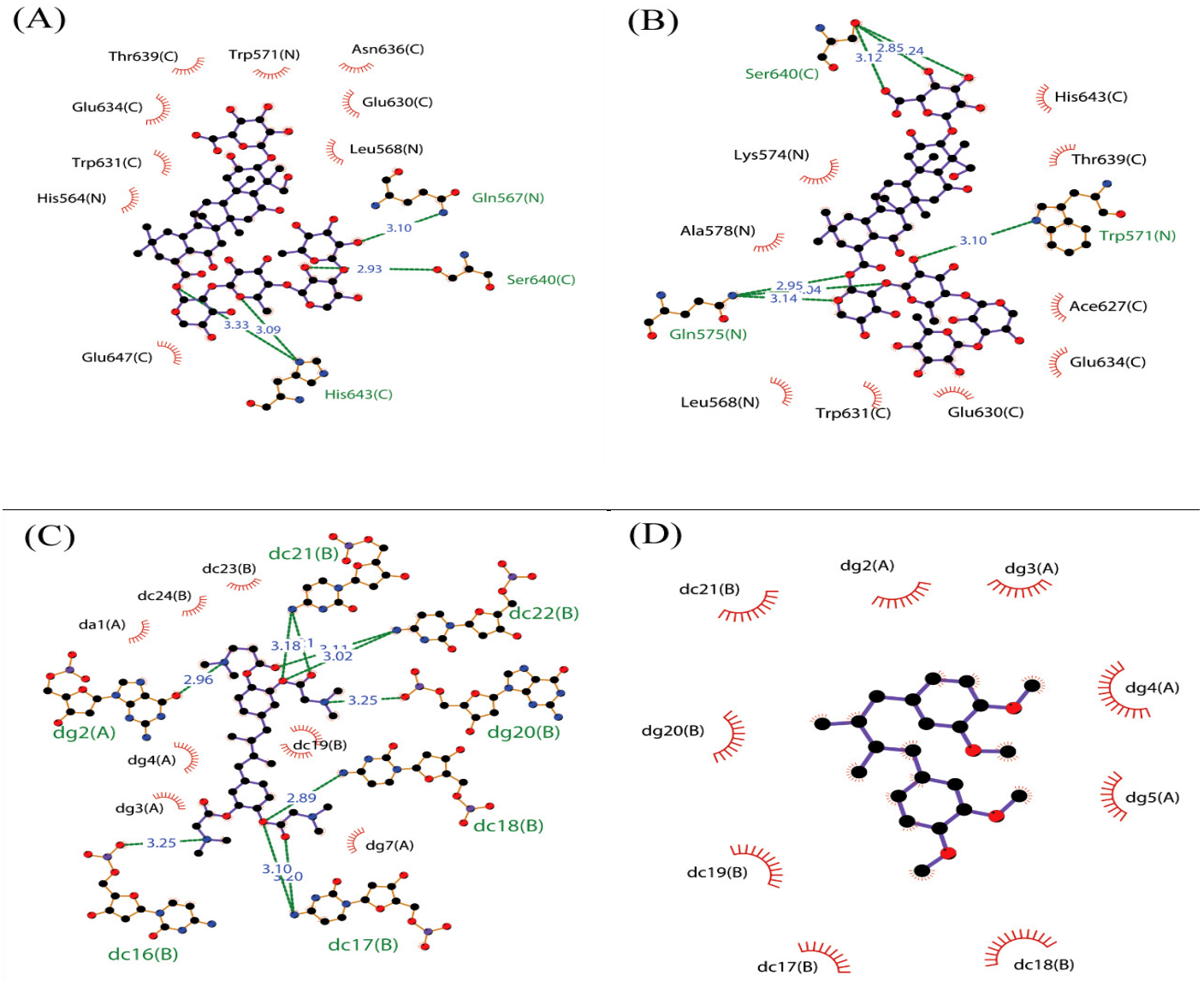

Figure 6. Hydrophobic interactions of Gen-1-bound gp41 **A and B)** and G4N **C)** and M4N **D)** Compounds bound to the Sp1-binding site. The H-bond interactions are displayed as green dotted lines, and the hydrophobic interactions with surrounding amino acids or DNA are shown as arcs with spokes radiating towards the inhibitor. These schematic diagrams were generated by the LigPlot+ software [66].

infected patients. The efficacies of the three inhibitors against viral replication were determined individually and in triple combination. The viral entry inhibitor Gen-1, the integrase inhibitor M522, and the proviral transcription inhibitor G4N suppressed replication of the clinical isolates in PBMCs with IC50 values of 65.0 ± 0.69 μM, 18.0 ± 0.66 μM, and 27.0 ± 1.65 μM, respectively (Figure 4, Table 1). We found that the combination of these three inhibitors inhibited viral replication of the clinical isolates from an HIV-infected patients with a very low IC50 value of 3.0 ± 1.28 μM compared to each drug alone. The median effect analysis of the three drug combinations showed a synergistic antiviral effect against the replication of clinical isolates from HIV-infected patients. This was demonstrated by the CI values of 0.164, 0.199, 0.258, and 0.313 at the 50%, 75%, 90%, and 95% effect levels, respectively (Table 2).

Overall, the results of the current study indicated that the viral replication of the drug-resistant strain of HIV and the clinical isolate from HIV-infected patients in H9 cells and PBMCs were strongly inhibited by the triple combinations more than by using each drug alone. Furthermore, they showed low IC50 values with synergistic antiviral effects.

Analyses of molecular interactions

Molecular bindings that showed the lowest binding free energy were selected for further conformation and interaction analyses. As can be seen in (Figure 5), the Gen-1 compound showed two possible conformations. We termed them the bend mode (A), which showed binding-free energy of -9.9 kcal/mol, and the straight mode (B), which showed binding-free energy of -9.2 kcal/mol. Binding conformation analysis revealed that in the bend mode, Gen-1 could form H-bond interactions between Gln567 of the N-heptad repeat (NHR) (3.10 Å), Ser640 of the C-heptad repeat (CHR) (2.93 Å), and His643 of CHR (3.09, 3.33 Å); whereas in the straight mode, Gen-1 could form H-bond interactions between Trp571 of NHR (3.10 Å) and Gln575 of NHR (2.95, 3.14, 3.04 Å). Hydrophobic interaction analyses revealed that the Gen-1 bend mode formed hydrophobic interactions with His564, Gln567, Leu568, Trp571, Glu630, Trp631, Glu634, Asn636, Thr639, Ser640, His643, and Glu647, whereas the Gen-1 straight mode showed hydrophobic interactions with Leu568, Trp571, Lys574, Gln575, Ala578, Glu630, Trp631, Glu634, Thr639, Ser640, and His643, as depicted in (Figures 6A and 6B), respectively.

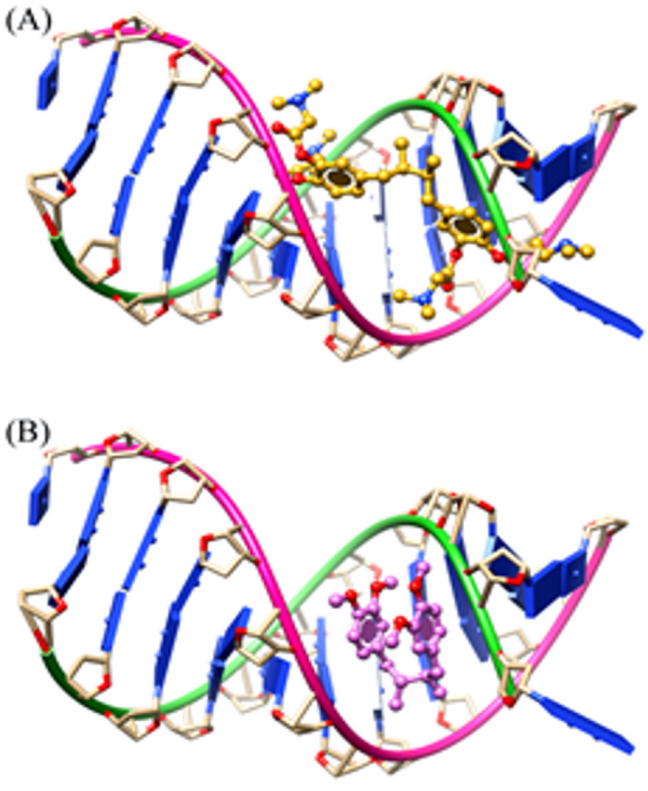

Figure 7. The binding conformation of G4N **A)** and M4N **B)** Bound to the Sp1-binding site. The DNA is shown as a red and blue helix, and the G4N and M4N are depicted in a ball and stick model. The figures were prepared using UCSF Chimera software version 1.11.2 [67] (to see this figure in colour, refer to the web version).

The binding poses of G4N and M4N have been analyzed and are shown in (Figure 7). We found that G4N showed binding-free energy of -10.4 kcal/mol and M4N -7.0 kcal/mol. (Figure 7A) shows that G4N was folded in the bend mode. It formed an H-bond interaction with C116, C117, C118, C221, C222, C223, and the G22 base pair. These H-bonds disappeared in the M4N compound. We also found that M4N was folded more compactly compared to G4N, as can be seen in (Figure 7B). This was due to a lower degree of freedom and resulted in the formation of a π…π interaction between two phenyl rings. Additionally, these two inhibitors induced DNA intercalation at the minor groove region. The tetra-O-glycyl-NDGA moiety of G4N can form manifold H-bonds with DNA. Furthermore, as shown in (Figure 6C), the (CH3)2 with adjacent positively charged nitrogen could form hydrophobic interactions with DNA. These hydrophobic interactions were conserved and found in M4N-bound DNA, as shown in (Figure 6D), which indicates the role of hydrophobic interactions for molecular binding.

The docking of M4N with the sequence CTGGGCGGGACT [38] that is present in the

LTR of the HIV-1 genome region shows the possibility of ligand (M4N) binding, as shown in (Figure S1A-C and S1E). We do not know how the target acts *in vivo* because the ligand M4N can reach the target. The docking energy and geometry are favorable, so if there are no circumstances that exclude the binding, the geometry itself is fine. The ligand M4N is not able to act as a hydrogen bond donor; thus, the interactions are limited to π…π interactions. The ligand M4N penetrates the binding site formed by the DNA bases and thus can modulate the action. While the docking of M4N with the sequence AGGGGCGGCGACT [39] that is present after the LTR region of the HIV-1 genome shows that the shape complementarity is very good, the ligand fits well into the pocket. In addition, a dimethoxy group accepts a hydrogen bond from a guanine that can strengthen the interaction. There is a π…π interaction between a dimethoxyphenyl group and another guanine that might strengthen this interaction. Docking energy is low, suggesting potential strong interaction. The docking of M522 with the sequence AGGGGCGGCGACT [39] that is present after the LTR region of the HIV-1 genome, as shown in (Figure S1) (A, D, E), indicates that the aromatic ring of the ligand M522 is partially intercalating into DNA double helices. The ligand being intercalated means that there are hydrophobic interactions between the ligand and DNA (specifically called the π…π interaction).

Discussion

Antiretroviral regimens approved for HIV treatment can be categorized into six major classes based on their activity and HIV life cycle target: 1) NRTIs, which are incorporated into the nascent HIV DNA chain and halt further DNA synthesis; 2) non-NRTIs, which bind directly to Reverse Transcriptase (RT) and interfere with its ability to synthesize viral DNA; 3) integrase inhibitors, which block the activity of the viral Integrase (IN) to insert HIV DNA into host chromosomes; 4) protease inhibitors, which inhibit the activity of the viral protease and block viral maturation; 5) fusion inhibitors; and 6) coreceptor antagonists [44]. Two of the inhibitors analyzed in this report, Gen-1 and M522, are new members of the HIV entry (fusion) and integrase classes of inhibitors, respectively. The third, transcription inhibitor and anticancer drug M4N, is a member of a new class of ART agents designated as HIV Tat-mediated transactivation inhibitors [45].

Proviral transcription from the HIV LTR promoter is a complex process that involves the cooperation of both viral and cellular factors. The viral protein Tat increases the production of elongated proviral transcripts through its interaction with the Trans-Activating Region (TAR) of the short, paused RNAs generated by the basal transcription machinery [46]. In the last two decades, various Tat-transactivation inhibitors have been identified [45] including RNA, peptide, and small-molecule TAR ligands and, more recently, compounds that interact directly with Tat to inhibit its function or accelerate its degradation. Methylated NDGA derivatives like M4N were found to inhibit basal HIV proviral

transcription and Tat-transactivated transcription *in vitro*. The presumed mechanism of action was interference with SP1 transcription factor binding to the HIV promoter [18,26]. This explanation does not easily explain M4N's effect on Tat-transactivation, although there is new evidence that Tat has a versatile role in regulating proviral transcription that may include interaction with Sp1 sites within the HIV promoter [47]. For this reason, Tat-transactivation inhibitors are also considered candidate agents for the suppression of HIV reactivation from its latent reservoir and, because of their nucleic acid targets (as for M4N and the TAR binding agents), might be expected to be less prone to inducing drug resistance. The hidden HIV-1 virus is integrated as a provirus within host cellular DNA [48].

The integrase inhibitors raltegravir, elvitegravir, and dolutegravir are the newest class of HIV ART drugs to be approved [7]. These drugs have been shown to be effective in ART-experienced patients harboring drug-resistant viruses, and because of their tolerability, better drug–drug interaction profile, dosing, excellent side-effect profiles, and high genetic barrier to resistance, they are now included in recommended regimens for ART-naïve patients [49]. We have recently shown that M522 (lithospermic acid) was effective in suppressing viral replication of HIV-1 strains resistant to raltegravir, structurally diverse PR inhibitors, and non-NRTIs. Furthermore, viruses exposed to increasing concentrations of M522 (four times its IC50 value) through 20 passages of selection (160 days) remained sensitive to the inhibitor [23].

Entry of HIV-1 into the host cell is a complex, multistage process that includes virus attachment, co-receptor binding, and membrane fusion [50]. The precise mechanism of action of Gen-1 as a viral entry inhibitor has yet to be elucidated. It is presumed to be a membrane fusion inhibitor as it was isolated using an HIV-1 envelope protein-mediated cell fusion assay. The target of Gen-1 could be the HIV-1 envelope or the host cell membrane.

Potent therapeutic effects against various viruses can interfere with the specific steps of the viral life cycle by blocking the binding between the virus and the host cells [10]. Combining a non-toxic human broad neutralizing antibody with antiretroviral drugs resulted in synergistic antiviral activity against diverse HIV-1 strains, including those resistant to NRTIs, as well as a delay in the emergence of drug-resistant strains and a reduction in drug toxicity [11]. N-p-Tosyl-L-Phenylalanine Chloromethyl Ketone (TPCK) inhibits HIV-1 replication in the late stages of its life cycle by impeding viral protease enzyme activity. Furthermore, the combination of TPCK with established HIV-1 protease inhibitors exhibits significant synergistic inhibitory potential, implying that TPCK could be used in a combination ART regimen [51]. In addition, compounds of HIV-1 integrase-LEDGF allosteric inhibitors retained high antiviral activity against HIV-1 variants resistant to IN strand transfer inhibitors [52]. It was found that no synergistic antiviral action was detected when AZT was combined with a palbociclib inhibitor [53]. Our non-toxic compounds, Gen-1 and M522 with either G4N or M4N, targeted three steps of the HIV life cycle and showed high inhibition effects of viral replication for the infected cells with the drug-resistant strain and the clinical isolate from HIV-infected patients. The efficacy of the three-drug combinations for these inhibitors against viral replication showed strong synergistic antiviral effects. Therefore, these drug combinations protected the new cells produced by cell division from reinfection with HIV-1.

Current lifelong ART can be disrupted by undesirable side effects or the development of drug resistance. Botanical drugs, because they are generally less expensive and have fewer side effects, are an attractive alternative to conventional pharmaceuticals for overcoming these obstacles [54]. The natural products calanolides (coumarins), betulinic acid (a triterpene), baicalin (a flavonoid), and polycitone A (an alkaloid) are promising anti-HIV agents that are currently being studied [55-58]. Similarly, Gen-1, M522, and M4N were also derived from medicinal plants, and while triterpenoid saponins can cause hemolysis, M522 and methylated NDGA derivatives have low toxicity and, as components of Danshen and Chaparral, have been extensively used by indigenous peoples of China and the desert Southwest for treating a variety of maladies [24,59]. Furthermore, both M522 (salvia salt) and M4N (terameprocol) have been studied in clinical trials: M522 for coronary artery disease [60] and M4N for advanced leukemias and high-grade glioma [28,43]. In these trials, M4N was well tolerated by patients at doses as high as 1700 mg/day.

Molecular docking calculations of Gen-1 revealed two possible conformations in which the C28 side chain sugar fragment of two conformations was placed and extensively formed H-bond and hydrophobic interactions, particularly with the C28-substituent that occupied the hydrophobic region of the NHR, as shown in (Figure 5). This confirmed the role of the C28 fragment in the antiviral activity of the Gen-1 molecule [61] and amino acids in the NHR region [62,63]. The D-Glc moiety of the Gen-1 bend mode was occupied and pointed toward Trp571. This conformation might form the CH…π interaction between CH of the D-Glc part and the aromatic ring of Trp571 by a distance of 2.75 Å (H to centroid center of Trp571). The core structure of Gen-1 straight mode formed a hydrophobic interaction with the backbone of Lys574. Moreover, the L-tRha (end of C28 substituent) was placed in proximity to Trp571 and formed an OH…π interaction by a distance of 3.07 Å [64]. The role of Trp571 and Lys574 in the molecular binding of the inhibitor has been reported by He and colleagues [62].

G4N and M4N were identified as inhibitors of the transcription factor Sp, which inhibited the growth of the HIV virus by blocking Sp1 from binding to a promoter of HIV genes [61]. We found that G4N occupied and inserted the tetra-O-glycyl-NDGA moiety through DNA, in which all parts of G4N are surrounded by DNA (Figure 6). The binding pose of M4N has likely characteristics with G4N, but only small parts contact and form interactions with DNA, which results in the lower binding free energy of ca. 3.4 kcal/mol as compared with G4N.

Our published data for the docking study showed that M522 binds HIV-1 integrase with the catalytic magnesium (Mg^{2+}) ion and forms metal-ligand chelation with nearby amino acids and viral DNA [65]. The 2,3-dihydrobenzofuran ring formed a π…π interaction with Tyr212, and one of the catechol moieties (R1) of M522 formed a π…π interaction with the adenosine base A17 of the viral DNA. Moreover, the hydroxyl oxygen atoms in catechol moieties can also form chelation interactions with Mg^{2+}. The second catechol ring (R2) of M522 established a hydrophobic interaction with Phe190 *via* an edge-to-face character [65-67].

The present docking calculations study of M4N has shown that the ligand (M4N) has the potential for strong interactions and binding with two different regions in the HIV-1 genome as described in the supplementary data (Figures S1A-C and S1E). The first M4N-binding DNA sequence (CTGGGCGGGACT) is found in the HIV-1 genome's LTR region [38], but the second M4N-binding DNA sequence (AGGGGCGGCGACT) is found after the LTR region [39]. While the docking calculations of M522 with the sequence that is present after the LTR region of the HIV-1 genome show that there are hydrophobic interactions between the ligand M522 and DNA (specifically called π…π interaction) (Figures S1A and S1D-E). We previously demonstrated that the M522 inhibitor binds to DNA sequences derived from the HIV-1 LTR region and inhibits 3'-processing of HIV-1 integrase and HIV-1 integrase catalytic activities by 3'-joining to the DNA (GACCCTTTTAGTCAGTGTGGAAAATCTCTAGCA) (Figure S1E), where the M522 inhibitor interacted with the catalytic core domain of HIV-1 IN and blocked its activity [17,23]. Therefore, M4N and M522 compounds have multiple binding sites in the HIV-1 genome, which is a new finding as shown in (Figure S2). This results in high inhibition of HIV-1 replication by a combination of these inhibitors, so that there is a significant synergy.

The cornerstone of successful HIV ART has been combination therapy that targets multiple steps in the viral life cycle. Additional targets and combination treatments directed at those targets would provide useful alternatives to current ART when long-term treatments become intolerable due to toxicity or drug resistance emerges due to undetected treatment failures. The high degree of synergy we observed for our three inhibitors confirmed their specificity and utility. They are involved in preventing viral entry, transcription, and integration to produce new virus particles [17,23] (Figure S2). The combination of an entry inhibitor, an integrase inhibitor, and a proviral transcription inhibitor has not previously been reported, and they can be examined with infected macrophages with HIV to test the latent virus. Since they are all botanical products derived from and non-cytotoxic, it may be predictive of their future success.

Conclusion

The drug combinations of Gen-1 and M522 with either G4N or M4N, which target three steps of the HIV life cycle (viral entry, integration, and transcription), inhibited viral replication in infected cells with the drug-resistant strain and the clinical isolates from HIV-infected patients. Our findings highlighted the efficacy of the triple combinations of these inhibitors against viral reproduction with their strong synergistic antiviral effects in all combinations, which are due to the different contributions of each drug. The docking calculations showed that Gen-1 can bind with the gp41 protein in two ways in which both interact with Trp571 and Lys574. The C28 side chain sugar fragment occupied the hydrophobic region of the NHR and formed numerous H-bond and hydrophobic interactions. G4N and M4N can occupy and energetically bind Sp1 *via* hydrophobic interaction, particularly the longer fragment of G4N's tetra-O-glycyl-NDGA moiety with the DNA minor groove. The potent transcription inhibitor M4N may be used with the drug combination of Gen-1 and M522 to inhibit the drug-resistant strain of HIV-1 in clinical trials. The three-drug combinations are potentially more effective for HIV-1 prevention than the single-drug treatment and are non-toxic, so they may serve as chemotherapeutic drugs for HIV-infected patients.

Authors' Contributions

ISA designed the study, carried out all the experiments on HIV, drug treatments, analyses, and interpretation of the study, conducted the last part of the docking study, and wrote the manuscript. KL and NN conducted the docking study. KL performed most of the docking analyses and wrote the docking section. RCCH supervised the study, revised, and edited the manuscript. All authors read and approved the final manuscript.

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Conflict of Interest

None.

References

1. Titanji, Boghuma Kabisen, Deenan Pillay and Clare Jolly. "Combination antiretroviral therapy and cell–cell spread of wild-type and drug-resistant human immunodeficiency virus-1." *J Gen Virol* 98 (2017): 821-834.
2. Mandal, Subhra, Michael Belshan, Ashley Holec and You Zhou, et al. "An enhanced emtricitabine-loaded long-acting nanoformulation for prevention or treatment of HIV infection." *Antimicrob Agents Chemotherapy* 61 (2017): 10-1128.
3. Kobayashi, Norihiko, Masako Nakahara, Masako Oka and Kumiko Saeki. "Additional attention to combination antiretroviral therapy-related lipodystrophy." *World J Virol* 6 (2017): 49.
4. Montaner, Julio SG, Viviane D. Lima, P. Richard Harrigan and Lillian Lourenço, et al. "Expansion of HAART coverage is associated with sustained decreases in HIV/AIDS morbidity, mortality and HIV transmission: The "HIV Treatment as Prevention" experience in a Canadian setting." *PloS One* 9 (2014): e87872.
5. Takamatsu, Yuki, Manabu Aoki, Haydar Bulut and Debananda Das, et al. "Novel protease inhibitors containing C-5-Modified bis-Tetrahydrofuranylurethane and Aminobenzothiazole as P2 and P2′ ligands that exert potent antiviral activity against highly multidrug-resistant HIV-1 with a high genetic barrier against the emergence of drug resistance." *Antimicrob Agents Chemotherapy* 63 (2019): 10-1128.
6. Meanwell, Nicholas A., Mark R. Krystal, Beata Nowicka-Sans and David R. Langley, et al. "Inhibitors of HIV-1 attachment: The discovery and development of temsavir and its prodrug fostemsavir." (2018): 62-80.
7. Council, Advisory. "Guidelines for the use of antiretroviral agents in adults and adolescents with HIV." *J Org Chem* 86 (2021): 693-708.
8. Podany, Anthony T., Kimberly K. Scarsi and Courtney V. Fletcher. "Comparative clinical pharmacokinetics and pharmacodynamics of HIV-1 integrase strand transfer inhibitors." *Clin Pharmacokinet* 56 (2017): 25-40.
9. Panel on Antiretroviral Guidelines for Adults and Adolescents. "Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents." Department of Health and Human Services (2011): 1-166.
10. De Clercq, Erik and Guangdi Li. "Approved antiviral drugs over the past 50 years." *Clin Microbiol Rev* 29 (2016): 695-747.
11. Wang, Xiaohuan, Peng Zou, Fan Wu and Lu Lu, et al. "Development of small-molecule viral inhibitors targeting various stages of the life cycle of emerging and re-emerging viruses." *Front Med* 11 (2017): 449-461.
12. Qi, Qianqian, Qian Wang, Weizao Chen and Fei Yu, et al. "Anti-HIV antibody and drug combinations exhibit synergistic activity against drug-resistant HIV-1 strains." *J Infect* 75 2017): 68-71.
13. De Pádua, Cristiane Menezes, Letícia Penna Braga Braga and Cássia Cristina Pinto Mendicino. "Adverse reactions to antiretroviral therapy: A prevalent concern." *Rev Panam Salud Publica* 41 (2018): e84.
14. Lu, Lu, Fei Yu, Lifeng Cai and Asim K Debnath, et al. "Development of small-molecule HIV entry inhibitors specifically targeting gp120 or gp41." *Curr Top Med Chem* 16 (2016): 1074-1090.
15. Mwimanzi, Philip, Ian Tietjen, Scott C. Miller and Aniqa Shahid, et al. "Novel acylguanidine-based inhibitor of HIV-1." *J Virol* 90 (2016): 9495-9508.
16. Rawson, Jonathan MO, Megan E. Roth, Jiashu Xie and Michele B. Daly, et al. "Synergistic reduction of HIV-1 infectivity by 5-azacytidine and inhibitors of ribonucleotide reductase." *Bioorg Med Chem* 24 (2016): 2410-2422.
17. Abd-Elazem, Ibrahim S., Hong S. Chen, Robert B. Bates and Ru Chih C. Huang. "Isolation of two highly potent and non-toxic inhibitors of human immunodeficiency virus type 1 (HIV-1) integrase from *S. miltiorrhiza*." *Antiviral Res* 55 (2002): 91-106.
18. Gnabre, John N., John N. Brady, David J. Clanton and Yoichiro Ito, et al. "Inhibition of human immunodeficiency virus type 1 transcription and replication by DNA sequence-selective plant lignans." Proc Natl Acad Sci U S A 92 (1995): 11239-11243.
19. Gosse, Benjamin, John Gnabre, Robert B. Bates and Christopher W. Dicus, et al. "Antiviral saponins from *T. heckelii*." *J Nat Prod* 65 (2002): 1942-1944.
20. Osbourn, Anne, Rebecca JM Goss and Robert A. Field. "The saponins–polar isoprenoids with important and diverse biological activities." *Nat Prod Rep* 28 (2011): 1261-1268.
21. Soetan, K. O., T. O. Ajibade and A. S. Akinrinde. "Saponins; A ubiquitous phytochemical: A review of its biochemical, physiological and pharmacological effects." *Recent Prog Med Plants* 43 (2014): 1-24.
22. Francis, George, Zohar Kerem, Harinder PS Makkar and Klaus Becker. "The biological action of saponins in animal systems: A review." *Br J Nutr* 88 (2002): 587-605.
23. Abd-Elazem, Ibrahim S. and Ru Chih C. Huang. "Inhibition of the replication of clinical drug-resistant HIV-1 strains by small molecule integrase inhibitors M522 and M532." (2014): 378.
24. Zhou, Limin, Zhong Zuo and Moses Sing Sum Chow. "Danshen: An overview of

its chemistry, pharmacology, pharmacokinetics and clinical use." *J Clin Pharmacol* 45 (2005): 1345-1359.

25. Wang, Jie, Xingjiang Xiong and Bo Feng. "Cardiovascular effects of salvianolic acid B." *Evid Based Complement Alternat Med Title* (2013): 247948.
26. Hwu, Jih Ru, Wen Nan Tseng, John Gnabre, Paul Giza and Ru Chih C. Huang. "Antiviral activities of methylated nordihydroguaiaretic acids. 1. Synthesis, structure identification and inhibition of tat-regulated HIV transactivation." *J Med Chem* 41 (1998): 2994-3000.
27. Huang, Ru Chih C., Yen Li, Paul E. Giza and John N. Gnabre, et al."Novel antiviral agent tetraglycylated nordihydroguaiaretic acid hydrochloride as a host-dependent viral inhibitor." *Antivir Res* 58 (2003): 57-64.
28. Grossman, Stuart A., Xiaobu Ye, David Peereboom and Myrna R. Rosenfeld, et al. "Phase I study of terameprocol in patients with recurrent high-grade glioma." *Neuro-Oncol* 14 (2012): 511-517.
29. Chou, Ting-Chao and Nick Martin. "CompuSyn for drug combinations and for general dose-effect analysis." *ComboSyn Inc Paramusn (NJ)* (2005).
30. Chou, Ting-Chao. "Drug combination studies and their synergy quantification using the Chou-Talalay method." *Cancer Res* 70 (2010): 440-446.
31. Frisch, Michael J., G. W. Trucks, H. B. Schlegel and G. E. Scuseria, et al. "Gaussian 03, Revision C. 02. Wallingford, CT: Gaussian." *Inc* (2004).
32. Chan, David C., Deborah Fass, James M. Berger and Peter S. Kim. "Core structure of gp41 from the HIV envelope glycoprotein." *Cell* 89 (1997): 263-273.
33. Dohm, Julie A., Ming-Hua Hsu, Jih-Ru Hwu and Ru Chih C. Huang,et al. "Influence of ions, hydration and the transcriptional inhibitor P4N on the conformations of the Sp1 binding site." *J Mol Biol* 349 (2005): 731-744.
34. Gasteiger, Johann and Mario Marsili. "Iterative partial equalization of orbital electronegativity—a rapid access to atomic charges." *Tetrahedron* 36 (1980): 3219-3228.
35. Trott, Oleg and Arthur J. Olson. "AutoDock Vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization and multithreading." *J Comput Chem* 31 (2010): 455-461.
36. Bikadi, Zsolt and Eszter Hazai. "Application of the PM6 semi-empirical method to modeling proteins enhances docking accuracy of AutoDock." *J Cheminform* 1 (2009): 1-16.
37. Halgren, Thomas A. "Merck molecular force field. I. Basis, form, scope, parameterization and performance of MMFF94." *J Comput Chem* 17 (1996): 490-519.
38. Butovskaya, Elena, Brahim Heddi, Blaž Bakalar and Sara N. Richter, et al. "Major G-quadruplex form of HIV-1 LTR reveals a (3+ 1) folding topology containing a stem-loop." *J Am Chem Soc* 140 (2018): 13654-13662.
39. Keane, Sarah C., Xiao Heng, Kun Lu and Siarhei Kharytonchyk, et al. "Structure of the HIV-1 RNA packaging signal." *Sci* 348 (2015): 917-921.
40. Morris, Garrett M., David S. Goodsell, Robert S. Halliday and Ruth Huey, et al. "Automated docking using a Lamarckian genetic algorithm and an empirical binding free energy function." *J Comput Chem* 19 (1998): 1639-1662.
41. Solis, Francisco J. and Roger J-B. Wets. "Minimization by random search techniques." *Math Oper Res* 6 (1981): 19-30.
42. Chen, Hongshan, Li Teng, Jian-Nong Li and Richard Park, et al. "Antiviral activities of methylated nordihydroguaiaretic acids. 2. Targeting herpes simplex virus replication by the mutation insensitive transcription inhibitor tetra-O-methyl-NDGA." *J Med Chem* 41 (1998): 3001-3007.
43. Tibes, R., K. T. McDonagh, L. Lekakis and J. M. Bogenberger, et al. "Phase I study of the novel Cdc2/CDK1 and AKT inhibitor terameprocol in patients with advanced leukemias." *Invest New Drugs* 33 (2015): 389-396.
44. Arts, Eric J. and Daria J. Hazuda. "HIV-1 antiretroviral drug therapy." *Cold Spring Harb Perspect Med* 2 (2012): a007161.
45. Tabarrini, Oriana, Jenny Desantis and Serena Massari. "Recent advances in the identification of Tat-mediated transactivation inhibitors: Progressing toward a functional cure of HIV." *Future Med Chem* 8 (2016): 421-442.
46. Ott, Melanie, Matthias Geyer and Qiang Zhou. "The control of HIV transcription: Keeping RNA polymerase II on track." *Cell Host Microbe* 10 (2011): 426-435.
47. Das, Atze T., Alex Harwig and Ben Berkhout. "The HIV-1 Tat protein has a versatile role in activating viral transcription." *J Virol* 85 (2011): 9506-9516.
48. Archin, Nancie M., Julia Marsh Sung, Carolina Garrido and Natalia Soriano-Sarabia, et al. "Eradicating HIV-1 infection: Seeking to clear a persistent pathogen." *Nat Rev Microbiol* 12 (2014): 750-764.
49. https://medbox.org/document/recommendation-on-integrase-inhibitor-use-in-antiretroviral-treatment-naive-hivinfected-individuals-from-the-hhs-panel-on-antiretroviral-guidelines-for-adults-and-adolescents
50. Wilen, Craig B., John C. Tilton and Robert W. Doms. "Molecular mechanisms of HIV entry." *Adv Exp Med Biol* (2011): 223-242.
51. Trivedi, Jay, Payel Ghosh and Debashis Mitra. "Np-Tosyl-L-Phenylalanine Chloromethyl Ketone (TPCK) inhibits HIV-1 by suppressing the activity of viral protease." *Biochem Biophys Res Commun* 527 (2020): 167-172.
52. Bonnard, Damien, Erwann Le Rouzic, Matthew R. Singer and Zhe Yu, et al. "Biological and structural analyses of new potent allosteric inhibitors of HIV-1 integrase." *Antimicrob Agents Chemother* 67 (2023): e00462-23.
53. Glumakova, Ksenia, Georgy Ivanov, Valeria Vedernikova and Lena Shyrokova, et al. "Nucleoside Analog 2′, 3′-Isopropylidene-5-Iodouridine as Novel Efficient Inhibitor of HIV-1." *Pharmaceutics* 15 (2023): 2389.
54. Kurapati, Kesava Rao V., Vaenkata S. Atluri, Thangavel Samikkannu and Gabriella Garcia, et al. "Natural products as anti-HIV agents and role in HIV-Associated Neurocognitive Disorders (HAND): A brief overview." *Front Microbial* 6 (2016): 1444.
55. Brahmachari, Goutam and Shyamal K Jash. "Naturally occurring calanolides: An update on their anti-HIV potential and total syntheses." *Recent Patents Biotechnol* 8 (2014): 3-16.
56. Aiken, Christopher and Chin Ho Chen. "Betulinic acid derivatives as HIV-1 antivirals." *Trends Mol Med* 11 (2005): 31-36.
57. Li, Bao Qun, Tao Fu, Yao Dongyan and Judy A. Mikovits, et al. "Flavonoid baicalin inhibits HIV-1 infection at the level of viral entry." *Biochem Biophys Res Commun* 276 (2000): 534-538.
58. Loya, Shoshana, Amira Rudi, Yoel Kashman and Amnon Hizi. "Polycitone A, a novel and potent general inhibitor of retroviral reverse transcriptases and cellular DNA polymerases." *Biochem J* 344 (1999): 85-92.
59. Gnabre, John, Robert Bates and Ru Chih Huang. "Creosote bush lignans for human disease treatment and prevention: Perspectives on combination therapy." *J Tradit Complement Med* 5 (2015): 119-126.
60. Qiong, Zhang, Liu Ai-Dong and Huang Yong-Sheng. "Clinical non-inferiority trial on treatment of coronary heart disease angina pectoris of Xin-blood stasis syndrome type with lyophilized Salvia salt of lithospermic acid powder for injection." *Chin J Integr Med* 12 (2006): 12-18.
61. https://patents.google.com/patent/WO2008088806A1/en
62. He, Xiao-Yang, Peng Zou, Jiayin Qiu and Ling Hou, et al. "Design, synthesis and biological evaluation of 3-substituted 2, 5-dimethyl-N-(3-(1H-tetrazol-5-yl) phenyl) pyrroles as novel potential HIV-1 gp41 inhibitors." *Bioorg Med Chem* 19 (2011): 6726-6734.
63. Jiang, Shibo, Srinivasa R. Tala, Hong Lu and Nader E. Abo-Dya, et al. "Design, synthesis and biological activity of novel 5-((arylfuran/1 H-pyrrol-2-yl) methylene)-2-thioxo-3-(3-(trifluoromethyl) phenyl) thiazolidin-4-ones as HIV-1 fusion inhibitors targeting gp41." *J Med Chem* 54 (2011): 572-579.
64. Mohan, Neetha, Kunduchi P. Vijayalakshmi, Nobuaki Koga and Cherumuttathu H. Suresh. "Comparison of aromatic NH··· π, OH··· π, and CH··· π interactions of alanine using MP2, CCSD and DFT methods." *J Comput Chem* 31 (2010): 2874-2882.
65. Nunthaboot, Nadtanet, Kiattisak Lugsanangarm, Sirirat Kokpol and Ibrahim S. Abd-Elazem. "Binding mode prediction of biologically active compounds from plant Salvia Miltiorrhiza as integrase inhibitor." *Bioinformation* 9 (2013): 426.
66. Laskowski, Roman A. and Mark B. Swindells. "LigPlot+: Multiple ligand–protein interaction diagrams for drug discovery." *J Chem Inf Model* (2011): 2778-2786.
67. Pettersen, Eric F., Thomas D. Goddard, Conrad C. Huang and Gregory S. Couch, et al. "UCSF Chimera—a visualization system for exploratory research and analysis." *J Comput Chem* 25 (2004): 1605-1612.

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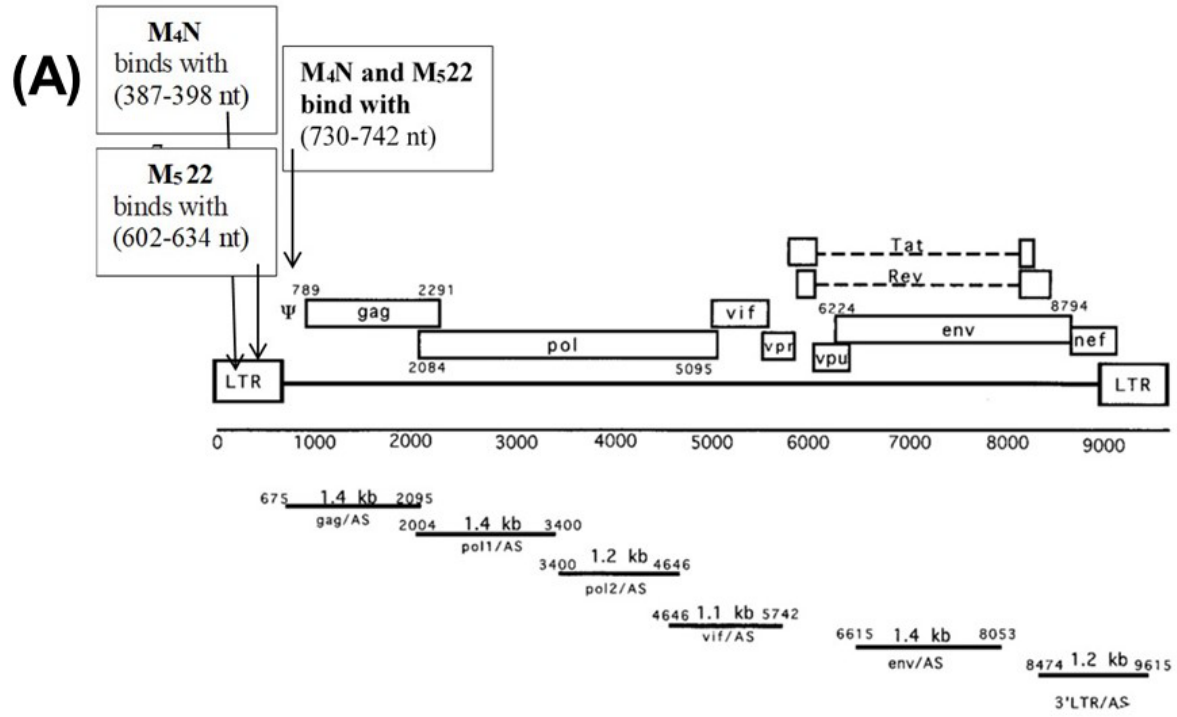
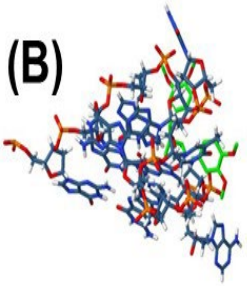
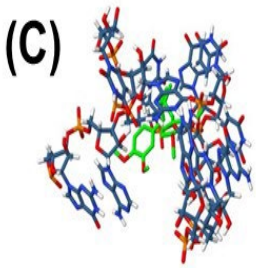
Appendix

Supplementary data

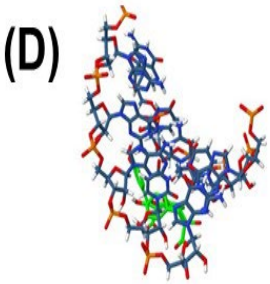




M_4N binds to CTGGGCGGGACT
(387-398 nt)



M_4N binds to AGGGGCGGCGACT
(730-742 nt)



M_522 binds to AGGGGCGGCGACT
(730-742 nt)

Figure S1. Schematic representation of the HIV-1 genome, docking of inhibitors M4N and M522, and HXB2 nucleotide sequence **A)** The nucleotide positions, sizes, and positions of the restriction fragments used for antisense-vector construction are indicated where our inhibitors M4N and M522 show the sites of binding (Veres, et al.). **B)** The docking of M4N with the sequence CTGGGCGGGACT that is present in the LTR of the HIV-1 genome region. The ligand M4N penetrates the binding site formed by the DNA bases. **C)** Docking of M4N with the sequence AGGGGCGGCGACT that is present after the LTR of the HIV-1 genome region shows that the dimethoxy group accepts a hydrogen bond from a guanine that can strengthen the interaction. **D)** The docking of M522 with the sequence AGGGGCGGCGACT, which is present after the LTR region of the HIV-1 genome, indicates the ligand M522 and DNA (specifically called the π...π interaction). All inhibitors are shown in blue and red, whereas the DNA bases are shown in green. **E)** (HIV-1 [HXB2], complete genome; HIV1/HTLV-III/LAV reference genome, GenBank: K03455.1).

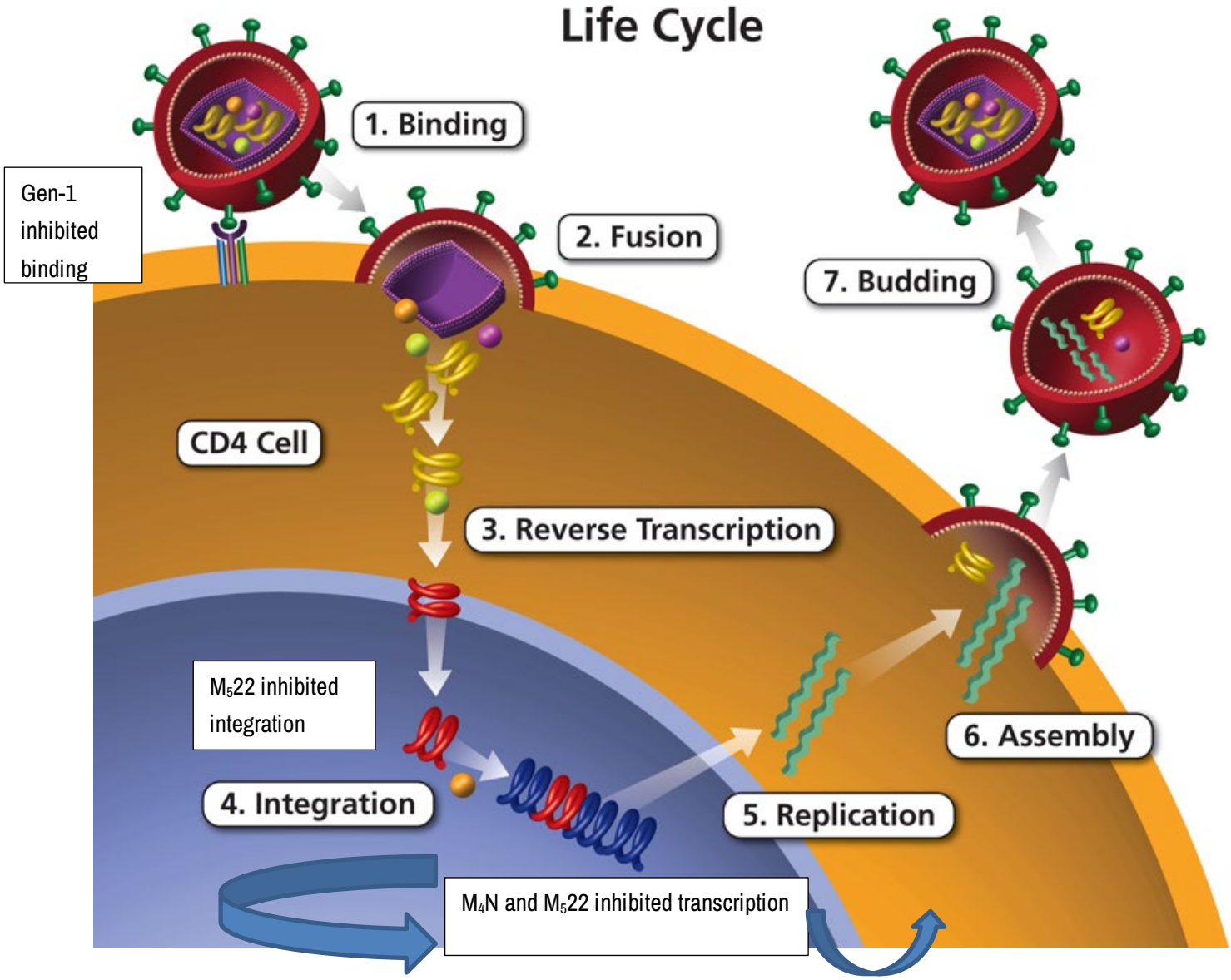


Figure S2. Replication cycle of HIV and drug targets by our inhibitors Gen-1, M522 and M4N. The life cycle of HIV is from the glossary of HIV/AIDS-Related Terms, 2021, 9th Edition (Clinical info.HIV.gov). The series of steps that HIV follows to replicate in the body. The process begins when HIV encounters a CD4 cell. The seven steps in the HIV life cycle are binding, fusion, reverse transcription, integration, transcription and replication, assembly, and budding. These are antiretroviral therapy targets, and our inhibitors Gen-1, M522, and M4N inhibit binding, integration, and transcription, respectively.

- Docking of M_4N inhibitor with the sequence CTGGGCGGGACT [1] that is present in the LTR region of the HIV-1 genome is equal to (387–398 nt) as in (Figure S1).
- Figure S1 shows M_4N inhibitor docking with the sequence AGGGGCGGCGACT [2], which is found in the ψ-gag region and follows the HIV-1 genome's LTR region (732–737 nt) [3].

- Docking of $M_5$22 inhibitor with the sequence AGGGGCGGCGACT [2], which follows the HIV-1 genome's LTR region (732–737 nt) and is present in the ψ-gag region [3] (Figure S1).
- Sequence that is present in the LTR region: CT**GGGCGG**GACT
- Sequence that is present in the gag region: G**GGGCGG**CGACT
- Both M_4N and $M_5$22 bind to the same sequence, **GGGCGG**, so that there is a significant synergy.
- The three-drug combinations of Gen-1 and $M_5$22 with either G_4N or M_4N, which target three steps of the HIV life cycle (viral entry, integration, and transcription), inhibited viral replication in cells infected with the drug-resistant strain and the clinical isolate from HIV-infected patients [4] (Figure S2).

Supplemental References

1. Butovskaya, Elena, Brahim Heddi, Blaž Bakalar and Sara N. Richter, et al. "Major G-quadruplex form of HIV-1 LTR reveals a (3+ 1) folding topology containing a stem-loop." *J Am Chem Soc* 140 (2018): 13654-13662.
2. Keane, Sarah C., Xiao Heng, Kun Lu and Siarhei Kharytonchyk, et al. "Structure of the HIV-1 RNA packaging signal." Science 348 (2015): 917-921.
3. Veres, Gabor, Uwe Junker, Jenny Baker and Carmen Barske, et al. "Comparative analyses of intracellularly expressed antisense RNAs as inhibitors of human immunodeficiency virus type 1 replication." *J Virol* 72 (1998): 1894-1901.
4. https://clinicalinfo.hiv.gov/sites/default/files/glossary/Glossary-English_HIVinfo.pdf