

Original Article

Antiretroviral drug-S for a possible HIV elimination

Agnieszka Agas¹, Heather Schuetz², Vikas Mishra¹, Adam M Szlachetka², James Haorah¹

¹Department of Biomedical Engineering, Center for Injury Bio Mechanics, Materials and Medicine, New Jersey Institute of Technology, Newark, NJ 07102, USA; ²Department of Pharmacology and Experimental Neuroscience, University of Nebraska Medical Center, Omaha, NE 68198, USA

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Abstract: Although the combination of highly active antiretroviral therapy (cART) can remarkably control human immunodeficiency virus type-1 (HIV-1) replication, it fails to cure HIV/AIDS disease. It is attributed to the incapability of cART to eliminate persistent HIV-1 contained in latent reservoirs in the central nervous system (CNS) and other tissue organs. Thus, withdrawal of cART causes rebound viral replication and resurgent of HIV/AIDS. The lack of success on non-ART approaches for elimination of HIV-1 include the targeted molecules not reaching the CNS, not adjusting well with drug-resistant mutants, or unable to eliminate all components of viral life cycle. Here, we show that our newly discovered Drug-S can effectively inhibit HIV-1 infection and persistence at the low concentration without causing any toxicity to neuroimmune cells. Our results suggest that Drug-S may have a direct effect on viral structure, prevent rebounding of HIV-1 infection, and arrest progression into acquired immunodeficiency syndrome. We also observed that Drug-S is capable of crossing the blood-brain barrier, suggesting a potential antiretroviral drug for elimination of CNS viral reservoirs and self-renewal of residual HIV-1. These results outlined the possible mechanism(s) of action of Drug-S as a novel antiretroviral drug for elimination of HIV-1 replication by interfering the virion structure.

Keywords: Human immunodeficiency virus, anti-retrovirus drug, macrophage, central nervous system

Introduction

Over the past decade, human immunodeficiency virus type-1 (HIV-1) has been one of the leading causes of death worldwide. With the emergence of combination antiretroviral therapy (cART), HIV-1 has become manageable and has transformed from a life-threatening to a life-long disease. Nevertheless, the Centers for Disease Control and Prevention shows an epidemic rise of new HIV/AIDS diagnoses in the United States among people living in urban areas (Centers for Disease Control and Prevention and [1]). Recent advances in cART has visibly reduced the level of replication, HIV-infection and HIV/AIDS progression [2]. This includes a better understanding of viral invasion in the brain, persistence, and neuropathogenesis [3-5]. Yet, HIV/AIDS disease remains without a cure even with this highly active ART. Additionally, the adverse side effects of ART are also known to cause neurological complications in HIV/AIDS patients such as dementia,

neuropathy, and various psychological conditions [6]. The efficacy of cART is further worsened by adverse drug interactions [7], co-infection [8], and malnutrition in HIV/AIDS patients because of inhibition of glucose/lipid metabolism known as lipodystrophy [9, 10]. HIV-1 infected cells are highly energy demanding due to enormous energy wasting and ART promotes malnutrition, which exacerbates metabolic imbalance in HIV/AIDS patients [11].

An equally important concern is the multi-faceted complications of HIV/AIDS progression in neuroAIDS patients with chronic substance abuse, where damage of the blood-brain barrier (BBB) enhances infiltration of infected cells into the brain that promotes neuroAIDS progression [12-14]. Thus, substance use is a risk factor for HIV-infection [15] resulting in the reduction of CD4+ T cells in HIV/AIDS patients. Credence of neuroAIDS in substance use shows depressive symptoms [16], loss of memory, increase neuropathy [17, 18], and excess mortality rates [19]

Novel antiviral agent for HIV elimination

among HIV/AIDS patients as well as increased predisposition to other health problems [20, 21]. We have shown that induction of oxidative stress, release of cytokines/glutamate in glial cells, and disruption of the BBB underlie the mechanisms of HIV-1 encephalitis, increased viremia, and neurotoxicity in the brain, which may contribute well to the progression of HIV/AIDS disease and behavior outcomes [22].

Multi-drug ART regimens are difficult to manage, but a three-drug combinational ART is needed to create a genetic barrier against drug resistance and viral mutations [23]. The development of fixed-dose, single tablet medications have reduced the complexity [24], but daily adherence can still be demanding [25]. Current ART research is focused on creating long-acting drugs that only require periodic injections [26], sustained release drugs with the use of nanoparticles [27], broadly neutralizing antibodies [28], and a safer less expensive alternative to those already available [29]. It is evident that ARTs can control viral replication, but a cure for HIV/AIDS is limited by less penetration of ARTs across the restricted anatomical features such as the BBB and enclosure within the restricted skull cavity [30-34]. Drugs that do cross the BBB are often thrown back out by way of saturable efflux systems such as the multi-drug resistant genes of endothelial cells [35]. Thus, a direct correlation of ART concentrations and HIV-1 viral load in the brain was observed [36]. Further, the blood-brain barrier is a bidirectional barrier, accumulated virions in the brain can re-enter the blood circulation for resurgence of HIV-1 infection [37]. This viral resurgence and inability of drugs to freely pass the BBB makes HIV/AIDS progression a constant threat.

The cure for HIV/AIDS disease is further diminished by the persistence of a viral reservoir in latently infected CD4⁺ T cell genotypes [38]. The stability of this latently infected HIV-1 reservoir is harbored in central memory CD4⁺ T cell genotype while the integrated HIV DNA is harbored in transitional memory CD4⁺ T cell genotype [39]. This was also observed in intravenous injection of HIV proteins into the brain of rats/mice [40]. Persistence of HIV-1 latency and CNS reservoir are responsible for the recurrence of HIV-1 self-renewal and reinfection upon withdrawal of ART in proliferating cells [41]. The ability of HIV-1 self-renewal and rein-

fection makes it a life-long dependency on ARTs and demonstrates that the body cannot completely clear itself of the virus. Longitudinal studies have shown that it would take someone on ARTs their lifetime to eliminate the virus [42]. However, with the continued successful outcome of the Berlin patient [43], there is new hope that a functional cure may be possible; where an infected person can suppress the virus to such low levels that taking ARTs is no longer a necessity. The Berlin patient showed mutations to the CCR5 gene from his hematopoietic stem cell transplant donor that effectively inhibited further infection [44].

Recently, several non-ART alternatives have been formulated for eliminating HIV-infection. Some of these non-ART alternatives are, gene editing to mutate the CCR5 gene [44], “shock-and-kill” approach to activate cells with latent reservoirs for immune cells to locate and kill [45], “block-and-lock” approach to length latency [46], immune modulation to boost the physiological immune system [47], viral-decay accelerators to increase mutation frequency towards catastrophe [48], hematopoietic stem cell transplants from infection resistant donors [44], pharmacological agents like microbicides to prevent transfer through sexual intercourse [49], microRNAs [50], and vaccines development approach [51, 52]. The shortcomings of these strategies include the non-feasibility for clinical applications [53], failure to reach the CNS [54], risks of inducing neurotoxicity [55] and other diseases, and these approaches cannot adjust for drug-resistant mutants [56]. Importantly, these strategies cannot eliminate all components of the viral life cycle as latent reservoirs hold replication incompetent HIV-1 that may contain provirus DNA, viral mRNA, viral protein, or any combination of these components [57]. Another major problem are viral mutations. Development of conformational changes in envelope glycoproteins due to viral mutations can mask or lose epitopes to evade immune attack [58], which presents another problem. With as many as 10 different mutants after one cycle of infection [59, 60], developing a comprehensive vaccine remains a great challenge.

With all these promising discoveries of active antiretroviral drugs, a critical need for a cure of HIV/AIDS disease will be to find a safe antiretroviral drug capable of inhibiting HIV-1 replication,

Novel antiviral agent for HIV elimination

eliminate self-renewal of residual HIV-1 in the CNS reservoir and peripheral latency. We report here that Drug-S has a potential to interfere with HIV-1 viral components, prevent rebound effect of HIV-1 self-renewal, and arrest HIV/AIDS progression. Drug-S is a natural substance derived from the roots of a plant that has not been tested or documented in the literature of ethnopharmacology. The plant extracts were used by indigenous people for mass poisoning using a large quantity of the extracts spiked into the drinks. Today, this same plant extract is used by the indigenous people for poisoning river fish for consumption. Consumption of the whole fish does not cause poisoning, perhaps due to low levels of toxin. Such dichotomy prompted the idea of whether this low concentration of toxin can inhibit HIV-1 infection and prevent the latently infected HIV-1 persistence without causing cytotoxicity.

Methods and materials

Reagents

The Drug-S extract was obtained by liquid-liquid extraction (water-methanol) of a plant root. The silica Bond Elut C18 extraction column and Vac Elut SPS 24 cartridge manifold used in solid phase extraction were purchased from Varian Medical Systems of Harbor City, CA. The Alltech C18 (4.6 × 100 mm, 5 µm) column and 25 mm VanGuard pre-column used in reverse-phase chromatographic separation was purchased from Waters Corporation of Milford, MA. Other common chemicals, reagents, and solvents used in the purification of Drug-S were purchased from Sigma-Aldrich of St. Louis, MO and were of the highest grade and purity.

Human monocytes were isolated by leukapheresis from HIV and hepatitis seronegative donors and differentiated to macrophage, microglia and neurons were isolated and purified from elective abortus specimens of human fetal brain tissues, while astrocytes and hBECs were purchased from ScienCell Research Laboratories of Carlsbad, CA. Tissues were obtained in full compliance with the ethical guidelines of the National Institutes of Health (NIH). The M-tropic strain HIV-1ada inoculum for infection were procured from NIH AIDS Reagent Program. Cell culture equipment, plastics, reagents, chemicals, and other supplies were purchased from Invitrogen of Carlsbad, CA, Thermo Fisher

Scientific of Waltham, MA, VWR of Radnor, PA, ScienCell Research Laboratories, or Sigma-Aldrich. Antibodies were purchased from Invitrogen, Thermo Fisher Scientific or Abcam of Cambridge, MA.

Primary cell isolation

Human monocytes were isolated from HIV and hepatitis seronegative donors and differentiated to macrophage, microglia and neurons were isolated from elective abortus specimens of human fetal brain tissues, while astrocytes and hBECs were purchased from ScienCell Research Laboratories. Tissues were obtained in full compliance with the ethical guidelines of the National Institutes of Health (NIH). Isolated cells cultured on cover slips in 24-well plates (40,000 cells/well) with respective cell culture media were assessed for cell purity by Iba-1 antibody for microglia, MAP-2 antibody for neurons, GFAP antibody for astrocytes, and von Willebrand factor/GLUT1 for hBECs, confirming 100% purity of all cell types.

Monocyte/macrophage culture

Human monocytes were isolated by leukapheresis from HIV and hepatitis seronegative donors and purified by counter-current centrifugal elutriation as previously described [40]. Monocytes, cultured at a density of 10⁶ cells/mL, were differentiated to macrophage in DMEM (Sigma Chemical Company) supplemented with 10% heat-inactivated pooled human serum, 50 µg/mL gentamicin, 10 µg/mL ciprofloxacin, and 20 ng/mL of macrophage colony stimulating factor (0.2% using a 10 µg/mL stock, Cell Signaling Technology, Cat. #8929) for 7 days to ensure adequate biological responses. Purity of macrophage was assessed by CD68 antibody (Abcam, 1:100) and showed 100% enrichment of macrophage.

Brain endothelial culture

Primary human brain microvascular endothelial cells (hBECs) and cell culture media were purchased from ScienCell Research Laboratories (Carlsbad, CA Cat. #1000, and #1001), and cells were cultured according to ScienCell's specifications. Briefly, cell culture plates, flasks, and glass cover slips were pre-coated with bovine plasma fibronectin (2 µg/cm² or 15 µg/mL in Dulbecco's phosphate buffered saline,

Novel antiviral agent for HIV elimination

Ca⁺⁺ and Mg⁺⁺-free), incubated at 37°C overnight, aspirated to remove excess fibronectin, and dried in a sterile hood overnight. Cells were seeded at a density of 9,000 cells/cm² on glass cover slips in 12-well plates for immunocytochemistry and in T-75 cm² flasks for protein extraction. Fresh cell culture media was changed every three days after seeding until the culture became fully confluent.

Astrocyte culture

Human astrocytes were purchased from ScienCell Research Laboratories (Cat. #1800) and cultured according to ScienCell's specifications. Briefly, cell culture plates, flasks, and glass cover slips were pre-coated with poly-L-lysine (2 µg/cm² or 15 µg/mL in double distilled sterile water), incubated at 37°C for 2 hours, aspirated to remove excess poly-L-lysine, rinsed with double distilled sterile water, and dried in a sterile hood overnight. Cells were seeded at a density of 5,000 cells/cm² on glass cover slips in 12-well plates for immunocytochemistry and in T-75 cm² flasks for protein extraction. Cell culture media, purchased from ScienCell Research Laboratories (AM, Cat. #1801), was refreshed the next day after initial seeding once a culture was established and changed every three days thereafter until the culture became fully confluent.

Microglia culture

Primary human microglia were isolated and purified from elective abortus specimens of human fetal brain tissues as described previously [61] and in accordance with the ethical guidelines of the National Institutes of Health (NIH). Briefly, dissociated tissues were digested with 0.25% trypsin-EDTA containing 0.0015% DNase for 30 min. at 37°C, neutralized with 10% fetal bovine serum in Hank's Balanced Salt Solution Ca⁺⁺ and Mg⁺⁺-free and further dissociated by trituration. The resulting single-cell suspension was cultured in DMEM (Sigma Chemical Company) supplemented with 10% fetal bovine serum, 1% l-glutamine, 1% ciprofloxacin and 0.2% gentamicin. After 14 days in culture, nonadherent microglia were collected and purified by preferential adhesion to resulting >98%-pure microglial cell populations. Cells were seeded at a density of 10,000 cells/cm² on glass cover slips in 12-well plates for immunocytochemistry. Purity of microglia was

assessed by Iba-1 antibody (Abcam, 1:100) and showed 100% enrichment of microglia.

Neuron culture

Primary human cortical neurons were isolated and obtained from elective abortus specimens of human fetal brain tissues as described previously [62-64] and in accordance with the ethical guidelines of the National Institutes of Health (NIH). Briefly, dissociated tissues were digested with 0.25% trypsin-EDTA containing 0.0015% DNase for 30 min. at 37°C, neutralized with 10% fetal bovine serum in Hank's Balanced Salt Solution Ca⁺⁺ and Mg⁺⁺-free, further dissociated by trituration, and filtered with 70 µm and 40 µm pore size strainers. Isolated neurons were seeded at a density of 50,000 cells/cm² on sterile glass cover slips pre-coated with poly-D-lysine (Neuvitro from Fisher Scientific) in 24-well plates for immunocytochemistry. Neurons were cultured in media containing Neurobasal™ Medium (Gibco, Cat. #21103049), 2% B-27™ (50X) serum-free supplemented with antioxidants (Gibco, Cat. #175-04044), 1% penicillin/streptomycin, and 0.2% l-glutamine, which was refreshed the next day after initial seeding once a culture was established and changed every three days thereafter. Purity of neurons was assessed by MAP-2 antibody (Invitrogen, 1:50) and showed 100% enrichment of neurons.

MTT assay

Cells cultured on 96-well plates were washed in PBS and incubated with tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (5 mg/mL MTT solution in 10% fetal bovine serum in PBS) for 45 mins at 37°C. After MTT solution was aspirated, plates were incubated with 100 µL dimethyl sulfoxide (DMSO) for 15 mins at room temperature. Conversion of water soluble 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to insoluble formazan was read spectrophotometrically at 490 nm absorbance.

Immunocytochemistry

Cells cultured on glass cover slips were washed with PBS, fixed in 4% paraformaldehyde for 15 mins, permeabilized with 0.1% Triton X-100 for 5 mins, blocked for unspecific binding with 3% bovine serum albumin for 1 hr. at room temper-

Novel antiviral agent for HIV elimination

ature, and incubated with respective primary antibodies such as rabbit anti-von Willebrand factor (vWF) (1:150 dilution), goat anti-Iba-1 (1:500 dilution), chicken anti-MAP2 (1:10,000 dilution), rabbit anti-GFAP (1:500 dilution), rabbit anti-CD68 (1:100 dilution), mouse anti-HIV1 p24 (1:1000 dilution) diluted in antibody buffer consisting of PBS, 1% BSA, and 0.4% Triton X-100 overnight at 4°C. After washing with PBS, cells were incubated for 1 hr. at room temperature with respective secondary antibodies: anti-rabbit-IgG Alexa fluor 594 for vWF, anti-goat-IgG Alexa fluor 488 for Iba-1, anti-chicken-IgG Alexa fluor 350 for MAP2, anti-rabbit-IgG Alexa fluor 594 for GFAP, anti-rabbit-IgG Alexa fluor 594 for CD68, and anti-mouse-IgG Alexa fluor 488 for HIV1 p24. Cover slips were mounted onto glass slides with Immuno Mount containing DAPI (Invitrogen). Images were captured by fluorescent microscopy (Eclipse TE2000-U, Nikon microscope, Melville, NY) using NIS elements (Nikon, Melville, NY) software.

Reverse transcriptase assay

Reverse transcriptase (RT) converts the single-stranded RNA genome to a cDNA molecule during active HIV-1 infection of host cells. HIV-1 virions were lysed with a lysis buffer solution (Bio-Rad Laboratories) to release RT. Supernatants from cell cultures were collected, clarified by low speed centrifugation, and pelleted by centrifugation at 100,000 g for 45 mins at 4°C to collect RT. Pellets were resuspended in 0.01 M Tris-HCl (pH 7.5) and sonicated for 40 sec. 10 µL of RT suspension was combined with a reaction mixture containing 0.05 M Tris-HCl buffer (pH 8.3), 0.06 M NaCl, 6 mM MnCl₂, 0.02 M dithiothreitol, 1 µg poly(rA), 0.5 µg oligo(dT), 0.05% Nonidet P-40, and 10⁻⁵ M [³H]dTTP (52 Ci/mmol) [65-67]. This reaction was incubated at 37°C for 20 mins, then stopped and analyzed for radioactivity by autoradiography (counts per million).

Purification of Drug-S

Methanol and water were used to extract the bioactive metabolite, Drug-S, from the bark of a plant root. This extract was evaporated by a rotary evaporator, re-dissolved in methanol, and subjected to solid phase extraction using a silica Bond Elut C18 extraction column (Varian Medical Systems) with a Vac Elut SPS 24 cartridge manifold (Varian Medical Systems) for sample preparation. Impurity was washed away

with hexane with remnant nonpolar impurities being removed by dichloromethane (DCM) eluent. DCM extract was concentrated by distillation and evaluated for recovery, cell toxicity, and antiretroviral activity. After observing high antiretroviral activity, this highly water-soluble DCM analyte was semi-purified by reverse-phase chromatographic separation using an Alltech C18 (4.6 × 100 mm, 5 µm) column (Waters Corporation) fitted with a 25 mm VanGuard pre-column (Waters Corporation). Mobile phase consisted of A: water and B: acetonitrile, both with 0.1% trifluoroacetic acid (TFA) (v/v). Separation was done using an isocratic elution of 20% B with a flow rate of 1 mL/min at 220 nm UV detection. A volume of 500 µL (1.0 mg/mL in 0.1% TFA) sample per injection was run for 80 minutes to collect five different fractions, F1 (2-8 mins), F2 (17-31 mins), F3 (31-50 mins), F4 (50-60 mins), and F5 (60-80 mins) in multiple runs (see **Figure 1**).

Determination of fractions containing antiretroviral activity

Each pooled fraction was evaporated to dryness under an argon gas stream, dissolved in saline to a concentration of 0.5 mg/mL, and assessed for antiviral activity with M-strain HIV-1 infected human macrophage in culture. Briefly, media from infected with/without drug fraction and uninfected control cultures of macrophage were collected at days 1, 3, 5, and 7 post-infection and assayed for radiolabeled reverse transcriptase (RT) activity to determine which fraction was most successful at inhibiting viral replication. To confirm assay results, cells were stained for CD68 and P24-Ag and we found that fraction 4 (F4) contained the active antiretroviral activity. Thus, in this work we termed the antiviral activity of fraction 4, as Drug-S.

Statistical analysis

Results obtained from all experiments were quantified and compared using ANOVA via Statview. For comparisons between multiple conditions, the initial statistical analyses were performed using one-way ANOVA, followed by Bonferroni's test for multiple comparisons. The difference was considered significant if the adjusted *P*-value was less than 0.05. To power the sample size appropriately, all preliminary data will be combined with the future experiments data for statistical analyses. Data in

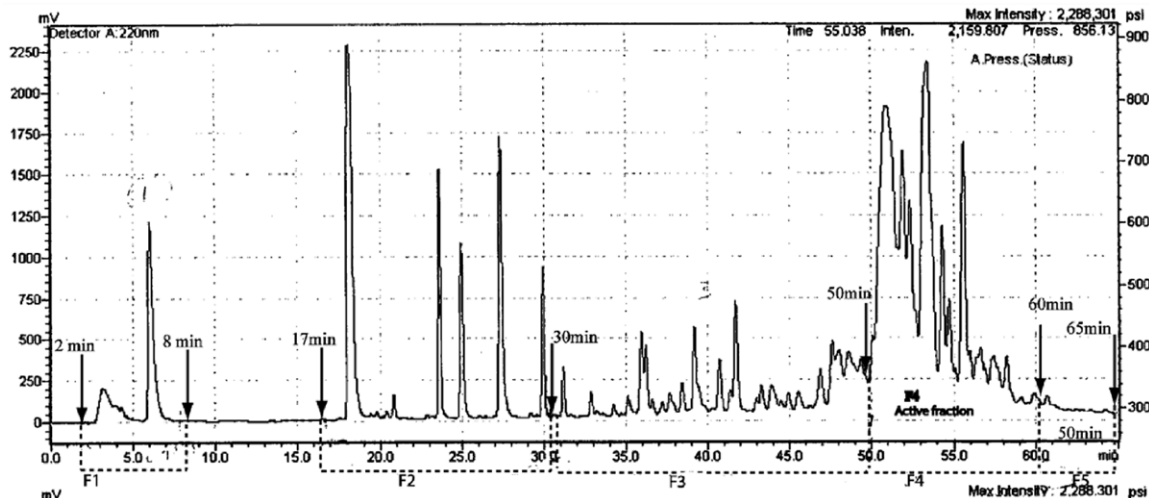


Figure 1. Representative graph of reverse-phase chromatographic separation fractions. Resulting peaks were collected into 4 fractions (F1-F5) each containing multiple peaks. Fractions are defined by two arrows and outlined by dotted lines and correspond to F1 being collect 2-8 mins into the run, F2 being collected 17-31 mins into the run, F3 being collected 31-50 mins into the run, F4 being collected 50-60 mins into the run, and F5 being collected 60-80 mins into the run, each in multiple runs. F5 was not analyzed because it did not contain peaks. F4 was found to contain the active antiretroviral activity termed Drug-S.

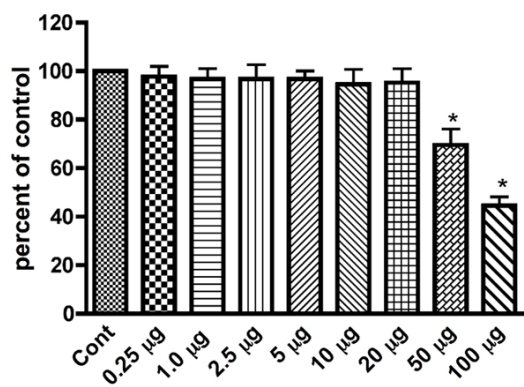


Figure 2. Dose-dependent toxicity of Drug-S in macrophage culture. Toxicity was assessed by MTT assay. Similar results were obtained for other cell types (data not shown). Drug-S caused significant cell death at concentrations higher than 20 µg/mL. However, concentrations below 20 µg/mL did not show toxicity.

mined the dose-dependent cytotoxicity of Drug-S in primary human macrophages, microglia, neurons, astrocytes and brain endothelial cells culture. The source, purity, and cell culture protocols of each cell type is described in methods. We first determined the effects of Drug-S from 0.25-100 µg/mL on cell viability by MTT assay in 96 well plate (1 × 204 cells/well). We established that Drug-S concentrations of 0.25-20 µg/mL were non-toxic to any of these primary cells, while concentrations of Drug-S higher than 20 µg/mL were toxic to all the tested primary cells in culture (see **Figure 2**). We observed a total inhibition of HIV-1 infection in macrophages at as low as 0.5 µg/mL of Drug-S same as that of 20 µg/mL (data not shown), as such we decided to use 0.5 µg/mL as our working concentration for inhibition of HIV-1 infection.

Drug-S inhibited HIV-1 infection

Human monocytes isolated by leukopheresis from HIV and hepatitis seronegative donors were purified by counter-current centrifugal elutriation. Monocytes culture at a density of 1 × 10⁶ cells/mL at 37°C were differentiated to macrophage in DMEM supplemented with 10% heat-inactivated pooled human serum, 1% glutamine, 50 µg/mL gentamicin, 10 µg/mL ciprofloxacin and 1000 U/mL recombinant human

graphs are shown as means ± SEM with N = 4, indicating actual number of samples or experiments performed and not the number of replicates per experimental condition.

Results

Dose-response of Drug-S

Since fraction 4 (Drug-S) was observed to contain highly active antiviral property, we deter-

Novel antiviral agent for HIV elimination

macrophage colony stimulating factor (MCSF) for 7 days to ensure adequate biological responses. Cells were infected with M-tropic strain HIV-1_{ada} inoculum for 16 hrs. After washing out the viral inoculum, infected cells were exposed to 0.5 µg/mL of Drug-S in fresh DMEM media (without MCSF) at 24 hr, 72 hr, day 5, and day 7 post infection. Cell culture media from HIV-1 infected with/without Drug-S, and uninfected control were collected at every 48 hrs before replacing with fresh media. Media supernatants were used for assessment of viral replication by RT assay, while cell culture on cover slips were stained for CD68, multinucleated giant cells (MGC), and HIV-1/P24-Ag staining. HIV-1 infection in the absence of Drug-S showed a number of MGCs and a massive HIV-1 P24-Ag positive staining (**Figure 3B** and **3E**) compared with infected cells in the presence of Drug-S (**Figure 3C** and **3F**) or uninfected cells (**Figure 3A** and **3D**). These results were further validated by RT activity to indicate that HIV-1 replication was inhibited by Drug-S. Indeed, RT activity at different time points in post infection showed an increase in HIV-1 replication in the absence of Drug-S compared with HIV-1 infection in the presence of Drug-S (**Figure 3G**). The absence of MGCs or HIV-1/P24-Ag staining in HIV infected cells (**Figure 3A-F**) and increase in HIV-1 replication (**Figure 3G**) in the presence of Drug-S indicated a clear inhibition of HIV-1 infection.

Drug-S pre-treated virions failed to infect macrophages

To assess if Drug-S can act directly on the virion structure, we pre-incubated cell free M-tropic strain of HIV-1_{ada} (0.01 MOI) with/without 0.5 µg/mL of Drug-S at 37°C for 1 hr prior to infecting macrophages for 16 hr. Supernatants collected in alternate days at post infection were assessed for neurotoxicity assay and RT activity. Our results showed that pre-treatment of HIV-1 virions with Drug-S failed to infect human macrophages (**Figure 4C**) same as uninfected control (**Figure 4A**) compared with HIV-1 infection without Drug-S (**Figure 4B**). It was interesting to observe that supernatants collected from Drug-S pre-treated HIV-1 virions did not cause neurotoxicity to human neuronal culture (**Figure 4F**) similar to supernatants collected from uninfected control (**Figure 4D**). As expected, HIV-1 virions without Drug-S pre-treatment showed significant neurotoxicity in culture (**Figure 4E**).

Assessment of viral replication further revealed a significant decrease in RT activity in Drug-S pre-treated HIV-1 virions (blue/black striped bars) compared with HIV-1 virions without Drug-S (pink/white striped bars) (see **Figure 4G**). Thus, pre-treatment of HIV-1 virions with 0.5 µg/mL of Drug-S prevented HIV-1 infection, suggesting that Drug-S may act directly on HIV-1 viral structure.

Drug-S inhibits latent HIV-1 persistence and reinfection

To establish HIV-1 elimination, supernatants were collected from HIV-infected macrophage at day 5 post-infection with/without Drug-S (0.5 mg/mL) or controls. These supernatants were added to macrophage or microglia culture in a separate set of experiments with 1:4 supernatant to fresh media. Supernatants from Drug-S treated showed no reinfection of macrophages or microglia, while supernatants from without Drug-S treatment cell culture showed reinfection in macrophage/microglia (**Figure 5**, top middle panel, shown for microglia).

In two separate experiments, macrophage culture in one plate were infected with HIV-1 in the absence of Drug-S, while the cells in second plate were infected with HIV-1 in the presence of 0.5 µg/mL of Drug-S. At day 3 post-infection, Drug-S was withdrawn from second plate and continued culturing in normal media. Thereafter, supernatants collected at day 1, 3, 5 and 7 were analyzed for viral replication by RT assay and cells on cover slips were analyzed for HIV-1 P24-Ag staining. Our results showed that withdrawal of Drug-S continued to prevent HIV-1 rebound infection (**Figure 5**, bottom panel). These results confirmed that Drug-S was capable of inhibiting the recurrence of HIV-1 persistence and reinfection.

Drug-S penetration across the BBB inhibited HIV-1 infection

To validate the proof-of-concept that Drug-S being a neurotoxin can cross the BBB, we examined the inhibition of HIV-1 infection in an *in vitro* models of BBB transwell cell to cell interactions. Human brain endothelial cells (hBECs) were first cultured on the luminal side while astrocytes were cultured on the abluminal side of transwells. Transwells membrane inserts with pore size of 0.2 µm were pre-coat-

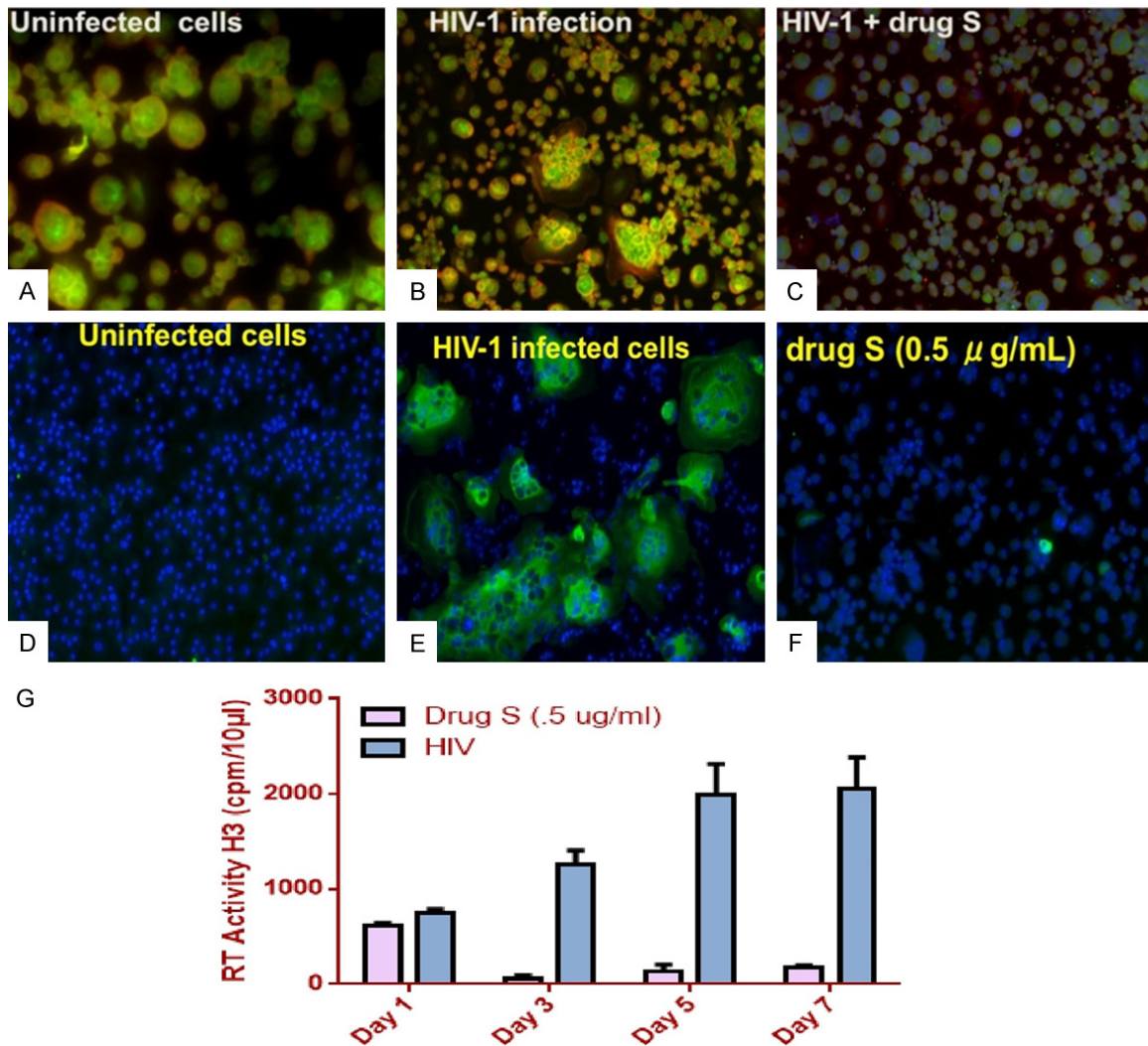


Figure 3. Drug-S inhibits HIV-1 infection in primary human macrophage. Upper panels = HIV infection (multinucleated giant cell clusters), middle panels = P24 staining, bottom bar graph = RT activity. One hallmark of HIV-1 infection in macrophage are the formation of multinucleated giant cells (upper panel, middle image) compared to uninfected control (upper panel, left image). In the presence of 0.5 μg/mL Drug-S, infected macrophage did not form multinucleated giant cells (upper panel, right image). Another indicator of HIV-1 infection is HIV-1/P24-Ag staining (middle panel, middle image) compared to uninfected control (middle panel, left image). P24 protein was detected by immunocytochemistry and fluorescent microscopy using specific antibody to P24 7 days post HIV infection. In the presence of 0.5 μg/mL Drug-S, infected macrophage did not stain for P24 (middle panel, right image). An RT activity assay (bottom bar graph) was conducted to determine HIV-1 replication in the presence (pink bar) and absence (dark shaded bar) of Drug-S. Media from infected with/without Drug-S and uninfected control cultures of macrophage were collected at days 1, 3, 5, and 7 post-infection and analyzed for presence of reverse transcriptase. Drug-S progressively lowered RT activity as compared to Drug-S untreated cultures that progressively increased activity. RT activity is expressed as cpms/10 μl of sample. Images are representative; original magnification was 20X. Data in the bar graph are presented as mean values ± SD (N = 4).

ed with rat-tail collagen and fibronectin to mimic the BBB basement membrane. Fully confluent hBECs and astrocytes in transwells (5 transwells/condition) were transferred to another plate that contained 16 hr HIV infected microglia at the bottom. Drug-S at the concentration of 2.0 μg/mL was applied on the luminal

side to examine whether Drug-S can cross the BBB components and inhibit HIV-1 replication in microglia at the bottom. Then cultured media from the bottom plates were collected at the time points shown in **Figure 6**. We found that application of Drug-S on the luminal side crossed the BBB and inhibited HIV-1 replication

Novel antiviral agent for HIV elimination

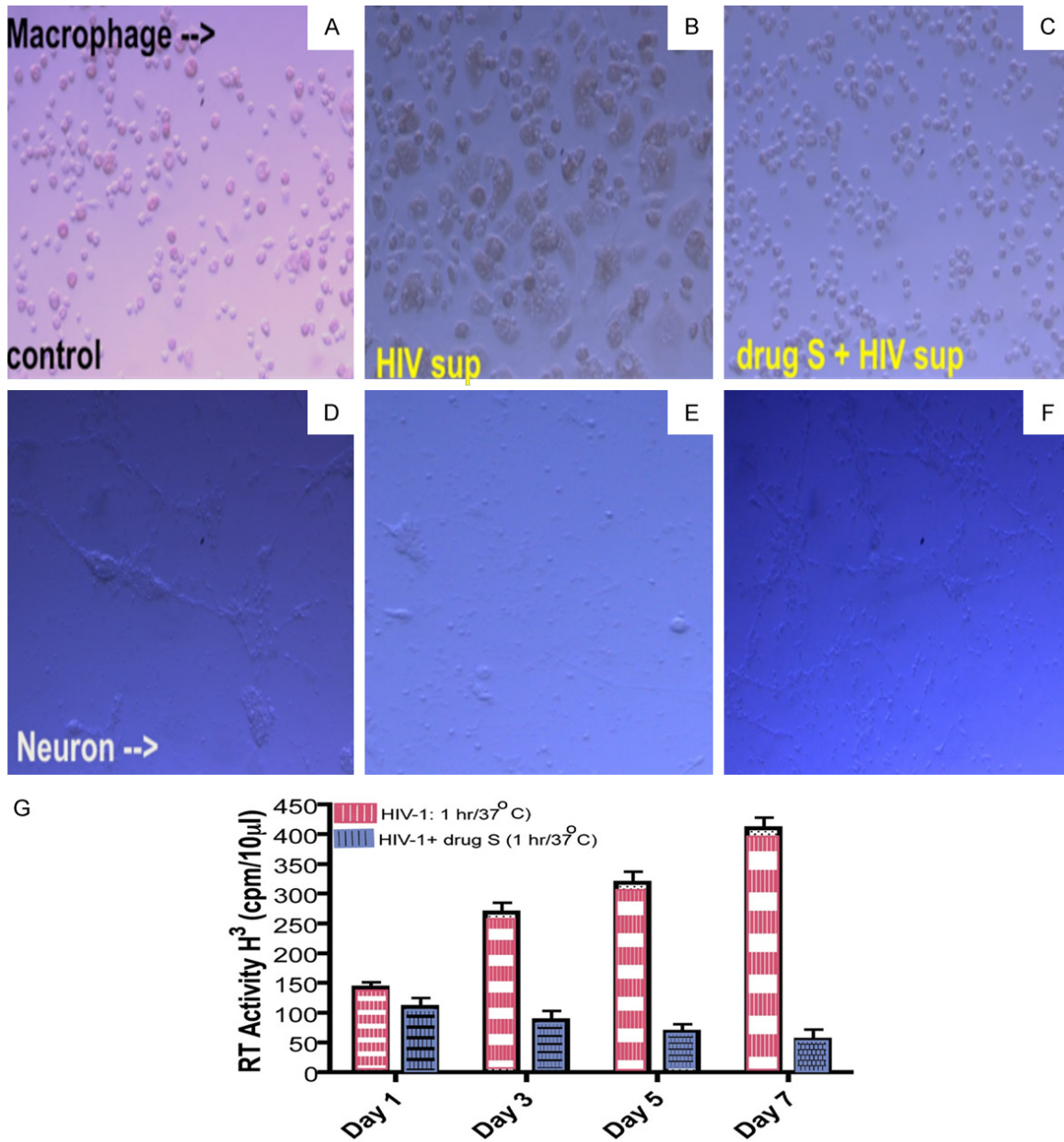


Figure 4. Drug-S pre-treated virions fail to infect primary human macrophages. Upper panels = HIV infection (multinucleated giant cell clusters), middle panels = neurotoxicity valuation (dead neurons), bottom bar graph = RT activity. Macrophage cultures were infected with HIV-1 virions pre-treated with/without 0.5 μ g/mL Drug-S for 1 hr. at 37 $^{\circ}$ C prior to infection. Virion pre-treatment with Drug-S prevented infection lacking formation of multinucleated giant cell clusters (upper panel, right image) as compared to cultures infected with Drug-S un-pre-treated virions (upper panel, middle image). Supernatants from HIV-1 virions with/without Drug-S pre-treatment and uninfected control were added to a culture of human neuron cells and analyzed for neurotoxicity. Neurons cultured with supernatants from control (middle panel, left image) and HIV-1 virions pre-treated with Drug-S (middle panel, right image) were not harmed compared with those cultured in supernatants from Drug-S un-pre-treated HIV-1 virions (middle panel, middle image). Healthy neurons exhibited neurite networks while dead neurons were rounded and lacked neurite networks. Media from cultures of macrophage supplemented with (pink bars)/without (black striped bars) Drug-S pre-treated virions were collected at days 1, 3, 5, and 7 post-infection and analyzed for presence of reverse transcriptase. Macrophage cultures supplemented with Drug-S treated virions showed no reinfection of macrophages, while virions without Drug-S treatment showed reinfection. RT activity is expressed as cps/10 μ l of sample. Images are representative; original magnification was 20X. Data in the bar graph are presented as mean values \pm SD (N = 4).

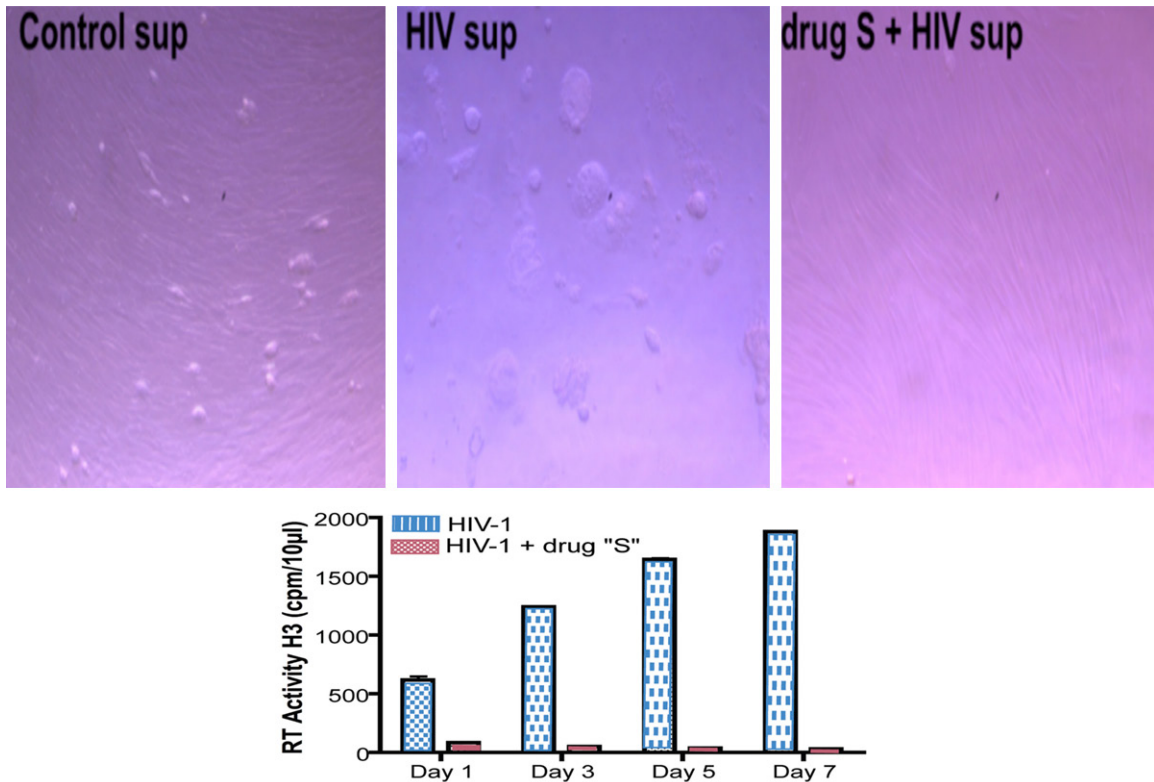


Figure 5. Drug-S inhibits latent HIV-1 persistence and reinfection. Upper panels = HIV infection (multinucleated giant cell clusters), bottom bar graph = RT activity. Re-infectivity of microglia occurred with supernatants from HIV-1 infected macrophage without Drug-S (upper panels, middle image), but not from HIV-1 infected macrophage in the presence of 0.5 µg/mL Drug S (upper panels, right image). Withdrawal of Drug-S at day 3 did not see a resurgence of RT activity (pink bars) in microglia unlike those infected without presence of Drug-S (checkered bars). RT activity is expressed as cpms/10 µl of sample. Images are representative; original magnification was 20X. Data in the bar graph are presented as mean values ± SD (N = 4).

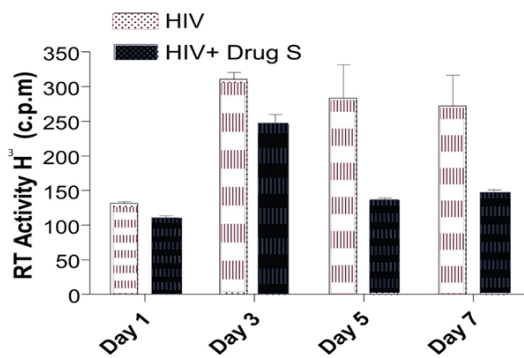


Figure 6. Drug-S penetration across the BBB inhibits HIV-1 infection. Drug-S readily crosses the BBB because addition of Drug-S (2.0 µg/mL) significantly decreased the RT activity in infected human microglia (black bars). RT activity is expressed as cpms/10 µL of sample and data are presented as mean values ± SD (n = 4).

in infected microglia (black bars) compared with HIV-1 infected microglia without Drug-S

(**Figure 6**). The presence of Drug-S continued to inhibit HIV-1 replication in microglia with time. These data suggest that Drug-S could cross the BBB readily and was able to arrest the progression of HIV-1 infection in neuroimmune cell like microglia.

Discussion

The rationale for this undertaking is that there is no ART capable of eradicating HIV-1 persistency and purging of the CNS-based latent HIV-1 reservoirs to-date. Our goal is to explore Drug-S as a potential candidate drug for a possible cure for HIV/AIDS disease, which possibility is in part substantiated by our data even though this wishful assumption is still premature. As such, the success of Drug-S as a viable antiviral drug requires more detail investigation. However, the use primary human macrophages, microglia, astrocytes, neurons and endothelial cells for testing the cytotoxicity of

Novel antiviral agent for HIV elimination

Drug-S in the present studies is appropriate since Drug-S appeared to be neurotoxin. The ability of Drug-S to inhibit HIV-1 infection and reinfection in human macrophage or microglia at the non-cytotoxic level has a significant scientific premise towards HIV/AIDS disease. Since lymphocytes are target of HIV-1 infection, we will also test the antiviral activity of Drug-S in HIV-1 infected human lymphocytes in future prior to pre-clinical testing.

Even though Drug-S (F4) contains high antiretroviral activity, Drug-S is still a pooled of multiple compounds in the present form, likely containing a number of impurities that will hinder the identification of Drug-S structure difficult. As such, we are now focusing on further purification of Drug-S active fraction for better evaluation of its antiviral activity, structure-activity relationships, and bio-distribution to bring a step closer to pre-clinical studies. Characterization of Drug-S is expected to enrich antiretroviral activity, decrease cytotoxicity, and be able to determine the chemical structure/mass. Separation of Drug-S purification will be achieved by preparative reverse-phase HPLC, determination of exact mass will be performed by high resolution MS, and identification of the structure of purified Drug-S will be determined by NMR, IR, and UV-Vis spectroscopy. Elucidating the molecular structure of Drug-S is also expected to unlock the ability to synthesize Drug-S that will no longer depend on harvesting plant products. It will also provide the possibility to fluorescently tag Drug-S and trace its movements inside a cell or animal.

With this challenging idea, the establishment of non-cytotoxic low concentrations of Drug-S (0.25-20 µg/mL) in human primary cell types so far tested is an important finding. Further, the effective inhibition of HIV-1 infection/reinfection, and arrest of HIV-1 replication by these non-cytotoxic low concentrations makes Drug S as a potential antiviral drug candidate for a possible elimination of HIV/AIDS. This argument is supported by our recent data of inhibiting HIV-1 infection/replication involving pre-treatment, withdrawal, and post-infection experiments. Yet, a comprehensive investigation on the mechanism(s) of HIV-1 inhibition is needed, which include the evaluation of Drug-S effects on HIV-1 virion structure, HIV-1 production during active infection, and host cellular

signaling pathways. Regardless, understanding the mechanism(s) of action of Drug-S may provide more information about HIV/AIDS progression that has not been gathered from traditional studies. Understanding the antiretroviral mechanism(s) of action of Drug-S will help guide Drug-S's development process, unacceptable drug combinations, and most importantly, this knowledge will allow Drug-S to be compared with available ART options.

The ultimate goal of Drug-S would be to compare or to work with already available ART drugs for a possible cure for HIV/AIDS disease. Persistence of HIV-1 latency in CNS reservoirs is the reason for reappearance of virus after withdrawal of ART, which compromises the possibility of HIV/AIDS eradication. A sterilizing cure for HIV-1 with clinical applicability, ability to penetrate the highly-protected CNS without causing neurotoxicity while retaining high antiretroviral activity has not yet been achieved. The innovation of this paper lies in presenting a new antiretroviral drug derived from a naturally occurring substance, which is capable of penetrating across the BBB and enclosure within the restricted skull cavity without causing neurotoxicity. It is expected to impact a good oral absorption and bio-distribution because Drug-S is highly stable hydrophilic compound. Above all, we have shown that Drug-S can effectively inhibit viral infection, replication, and self-renewal. This will be the first antiretroviral drug to act directly on the HIV-1 virion and eliminate the virus alone.

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Disclosure of conflict of interest

None.

Abbreviations

cART, combinational highly active antiretroviral therapy; HIV-1, human immunodeficiency virus type-1; HIV/AIDS, human immunodeficiency virus acquired immunodeficiency syndrome; CNS, central nervous system; neuroAIDS, neurological disorders caused by HIV; BBB, blood-brain barrier.

Novel antiviral agent for HIV elimination

Address correspondence to: Dr. James Haorah, Department of Biomedical Engineering, Center for Injury Bio Mechanics, Materials and Medicine, New Jersey Institute of Technology, CHEN Building, Room 120, 111 Lock Street, University Heights Newark, NJ 07102-1982, USA. Tel: 973-596-6595; Fax: 973-596-5222; E-mail: jhaorah@njit.edu

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