博士論文

Novel human immunodeficiency virus type-1 (HIV-1) inhibitors that reduce virus production via inhibition of viral Gag-host TSG101 interaction

（ヒト免疫不全ウィルス 1 型（HIV-1) Gag タンパク質と宿主因子TSG101との相互作用を標的とする新規HIV薬の開発）

Ph.D. Thesis

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Abbreviations

HIV-1	: human Immunodeficiency Virus Type-1
AIDS	: acquired Immunodeficiency Syndrome
ENV	: envelope
LTR	: long-terminal repeats
Pol	: polymerase
PR	: protease
MA	: matrix
CA	: capsid
NC	: nucleocapsid
SP1 and SP2	: spacer peptides 1 and 2
RT	: reverse transcriptase
IN	: integrase
SU	: surface envelope glycoprotein
TM	: transmembrane envelope glycoprotein
ssRNA	: single-stranded RNA
CXCR4	: chemokine receptor 4
CCR5	: CC-chemokine receptor 5
dsDNA	: double-stranded DNA
PIC	: pre-integration complex
HAART	: highly active antiretroviral therapy
ESCRT	: endosomal sorting complexes required for transport
PTAP	: Pro-Thr-Ala-Pro motif
TSG101	: tumor suppressor gene 101

$LYPX_nL$: Leu-Tyr-Pro-Xaa_n-Leu

ALIX : ALG-2(apoptosis-linked gene 2)-interacting protein X

Hrs : hepatocyte growth factor-regulated tyrosine kinase substrate

UEV : ubiquitin E2 variant

VLP : virus-like particles

DMSO : dimethyl sulfoxide

ELISA : enzyme-linked immunosorbent assay

GST : Glutathione S-Transferase

PBS : phosphate buffered saline

BSA : bovine serum albumin

HRP : horseradish peroxidase

TMB : tetramethylbenzidine

mAb : monoclonal antibody

HTS : high throughput screening

WST-1 : water-soluble tetrazolium salt-1

CC50 : 50% cytotoxic concentration

IC50 : 50% infectivity concentration

S/N : signal to noise ratio

S/B : signal to background ratio

pAb : polyclonal antibody

MOE : Molecular Operating Environment

SDS-PAGE : sodium dodecyl sulfate-polyacrylamide gel electrophoresis

PVDF : polyvinyldene difluoride

EC50 : 50% effective concentration

Introduction

Human Immunodeficiency Virus Type-1

The human immunodeficiency virus type-1 (HIV-1) is a lentivirus belonging to the Family *Retroviridae*. Like other retroviruses, this enveloped virus replicates in the host cell by reverse transcription, converting its single stranded RNA into a viral DNA with the catalytic activity of the viral enzyme reverse transcriptase (Cullen 1991). It mainly targets the cells of the human immune system such as $CD4^+$ T-helper cells, dendritic cells and macrophages (Freed and Martin 2007). As a lentivirus, it affects slowly and requires a long incubation period leading to a progressive failure of the immune system. The infection develops into acquired immunodeficiency syndrome (AIDS) and poses a great risk to acquire opportunistic infections and cancers.

The HIV-1 genome is composed of three major genes; *gag, pol* and *env*, which encode for structural proteins, viral enzymes and envelope (Env) glycoproteins of the virion, respectively (Cullen, 1991; Frankel and Young, 1998; Freed 2001; Watts *et al.,* 2009) (Figure 1A). It is flanked by two long-terminal repeats (LTR) necessary for reverse transcription, integration and gene expression steps (Suzuki and Suzuki 2011). It also contains four accessory genes namely, *vif, vpr, vpu* and *nef* and regulatory genes, *rev* and *tat*, which play important roles in transcription, RNA processing, virion assembly, host gene expression, and other replication functions (Cullen, 1991; Frankel and Young, 1998; Goff 2001). By subsequent proteolytic processing during virion maturation, Gag ($Pr55^{Gag}$), polymerase (Pol) ($Pr160^{GagPol}$) and Env glycoprotein (gp160) precursor polyproteins are cleaved into individual proteins (Freed 2001). $Pr55^{Gag}$ polyprotein is cleaved by the viral protease (PR) into Gag proteins matrix (MA or p17), capsid (CA or p24), nucleocapsid (NC or p7) and p6 along with two spacer peptides (SP1 and SP2), which altogether form the core of the virion (Frankel and

Young, 1998; Watts *et al*., 2009). Pol precursor polyprotein, $Pr160^{GagPol}$ cleaved into viral enzymes; reverse transcriptase (RT), integrase (IN) and protease (PR), which play significant enzymatic roles during reverse transcription of viral RNA to DNA, integration of viral genome to host genome and maturation of the progeny virion, respectively. Cleavage of gp160 Env precursor protein generates surface Env glycoprotein (SU or gp120) and transmembrane protein (TM or gp41) serve as outer membrane envelope of the virion (Frankel and Young, 1998; Freed 2001).

In addition to the viral proteins, the HIV-1 virion (Figure 1B) contains two copies of single-stranded RNA (ssRNA), in which together with other small RNAs constitute about 2.5% of the virion mass. Most of its mass comprised of the individual Gag proteins about 50%, 30% from viral membrane lipids and 20% from other viral and cellular proteins (Sundquist & Kräusslich, 2012). Typical among retroviruses, HIV-1 progeny virion is released as spherical immature virion from the infected cell and matures by proteolytic processing of its precursor proteins (Goff 2001).

HIV-1 Replication Cycle

HIV-1 replication begins with the virus attachment to the CD4 receptor and CD4 co-receptors: chemokine receptor 4 (CXCR4) and CC-chemokine receptor 5 (CCR5) in susceptible cells (Figure 2). CXCR4-using virus is known as X4-tropic HIV-1 while CCR5-using virus is known as R5-tropic HIV-1 (Freed and Martin 2007; Barré-Sinoussi *et al*., 2013). Upon successful attachment, the viral membrane and host's plasma membrane fused, leading to the uncoating of the viral capsid and the release of HIV ssRNA and viral proteins to the cytoplasm. By reverse transcription, viral ssRNA is transcribed into double-stranded DNA (dsDNA), and is imported to the nucleus along with viral proteins and a number of cellular proteins in a complex called pre-integration complex (PIC) (Suzuki and Craigie

2007). Viral dsDNA are then integrated into the host's chromosomal DNA, an indispensable step for efficient RNA transcription and production of infectious particle (Cullin 1991; Freed and Martin 2007; Schwartzberg, Colicelli and Goff 1984). New viral RNAs are synthesized and are exported from the nucleus to the cytoplasm through Rev proteins. These RNA transcripts are translated into precursor proteins, Gag ($Pr55^{Gag}$), Gag-Pol and Env as well as into accessory proteins (Freed 2015). These precursor polyproteins are targeted to the plasma membrane along with the viral RNA genome to promote virion assembly and budding mainly facilitated by Gag protein. An immature progeny virion is released from the infected cell and matures by proteolytic cleavage. Maturation step generates infectious progeny virion (Barré-Sinoussi *et al*., 2013).

Current Antiretroviral Treatment: HAART

It has been more than three decades since HIV-1 was identified as the causative agent of AIDS. Since then, numerous studies have already been conducted in the attempt to find a curative therapy. Yet, there is neither an effective vaccine available to prevent HIV-1 infection nor an effective cure as of to date to completely eradicate the virus from an infected person (Jiang 2011). The current anti-HIV-1 treatment commonly known as highly active antiretroviral therapy (HAART) is a combination of antiretroviral drugs, which targets viral proteins involved in viral entry and fusion as well as the key enzymes involved in each stage of HIV-1 life cycle such as reverse transcriptase, and viral protease (Kim 2011) (Figure 2). With persistent treatment through HAART, HIV-1 levels in infected patients are almost undetectable. This has led to a significant decline in HIV-1 related morbidity and mortality since HAART was introduced. However, there have been a number of drawbacks with this treatment such as significant side effects, emergence of drug resistant strains and the need to continue the treatment throughout the patient's lifetime. Moreover, HAART is unable to

activate the latent HIV-1 in resting T-cells and consequently, cannot completely eradicate the virus from the infected person (Arhel 2010). Hence, there is still a pressing need to search and develop a more potent antiretroviral drugs with novel targets and mechanisms of action to attain sustainable treatment.

HIV-1 Assembly, Budding and Release

A new target that can be used to develop novel antiviral agents has to be identified. One such target is the late stage of HIV-1 replication cycle, which includes HIV-1 assembly, budding and release. The assembly, budding, and release of new HIV-1 virions from the infected host cells involves complex interactions between the viral RNA genome, its viral proteins and the usurped host cellular factors, leading to virion budding through the plasma membrane and the subsequent fission event separating the virion from the infected cell (Martin-Serrano and Neil 2011) (Figure 3).

In detail, at the late stage of HIV-1 replication, $Pr55^{Gag}$ precursor protein contains 4 domains: matrix, capsid, nucleocapsid and p6 and 2 spacer peptides, SP1 and SP2 with each domain playing a significant role in the viral assembly and release (Figure 4) (Scarlata and Carter 2003; Freed 2015). MA domain is involved in targeting Gag to the plasma membrane, the site of assembly, as well as in promoting the incorporation of Env glycoproteins into the new virions. CA domain promotes the multimerization of Gag during assembly. NC domain recruits the viral RNA genome into the new virions and facilitates the assembly process. The main role of p6 domain is to recruit the endosomal sorting complexes required for transport (ESCRT) machinery for efficient membrane fission and release of the virions in an energy-depemdent process (Scarlata and Carter 2003; Tritel and Resh 2001; Freed 2015).

HIV-1 Gag p6 interacts with a number of host proteins, collectively known as ESCRT machinery to successfully egress from the infected cell (Figure 4). For HIV-1 budding to

occur, Gag proteins utilize the host' ESCRT machinery, which consists of four complexes (ESCRT-0, ESCRT-I, ESCRT-II and ESCRT-III). Specifically, the Pro-Thr-Ala-Pro (PTAP) motif of Gag p6 domain also known as late domain that is also found in most retroviruses, recruits the ESCRT-I by binding to Tumor suppressor gene 101 (TSG101) and another Gag p6 motif/late domain, the Leu-Tyr-Pro-Xaa_n-Leu ($LYPX_nL$) recruits the ESCRT-III by binding to ALG-2 (apoptosis-linked gene 2)-interacting protein X (ALIX) (Demirov and Freed 2004; Martin-Serrano *et al*., 2001; Usami *et al*., 2009) (Figure 4). PTAP-TSG101 mediated budding is the primary pathway used by HIV-1. TSG101 is a cellular protein subunit of ESCRT-I complex that normally functions in sorting proteins into vesicles, which bud into multivesicular bodies (von Schwedler *et. al*., 2003; Garrus *et al*., 2001). In normal cells, TSG101 is recruited by human hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) protein through the specific binding of its NH2-terminal ubiquitin E2 variant (UEV) domain to the Hrs PSAP motif to sort protein cargos and form multivesicular bodies, while in virus infected cells, the Gag p6 domain imitates Hrs activity (Pornillos *et al*., 2003) through its highly conserved PTAP motif and specifically binds to TSG101 also via UEV domain to facilitate the release of infectious virus particles from the plasma membrane (Im *et al*., 2010, Goff *et al*., 2003, Garrus *et al*., 2001, Kim *et al*., 2011 and VerPlank *et al*., 2001) (Figure 4). It is also notable that efficient release of virus particles occurs when TSG101 is recruited by Gag p6 to the HIV-1 budding site at the plasma membrane (Pornillos *et al*., 2002).

Gag-TSG101 Interaction and its importance

Gag PTAP-TSG101 interaction is indispensable for the proteolytic processing of Gag and for the efficient release of virus (Garrus *et al.,* 2001, Martin-Serrano *et al.,* 2001, VerPlank *et al.*, 2001). Several studies reported the effects of interfering Gag-TSG101 binding on viral

budding and release by deletion, mutation or overexpression of Gag or TSG101. Mutation of PTAP motif of Gag p6 or depletion of TSG101 blocks HIV-1 budding showing isolated or clustered immature virions still attached to the plasma membrane (Gottlinger *et al*., 1991, Im *et al*., 2010, Garrus *et al*., 2001 and Demirov *et al*., 2002), while deletion of either PTAP or TSG101 inhibited virus-like particle (VLP) release, with the latter having a greater extent of inhibition (Goff *et al*., 2003). Inhibition or overexpression of TSG101 not only significantly impaired viral production but also reduced the infectivity of the virus being produced (Garrus *et al*., 2001). A severe reduction on the infectivity of released virus is also observed when PTAP motif is disrupted (Usami *et al*., 2008). These indicate that PTAP-TSG101 interaction is not only required for efficient viral release but also for the release of infectious virus. Thus, interfering this Gag-TSG101 interaction provides a promising opportunity to identify HIV-1 replication inhibitors with novel mechanisms.

HIV-1 Budding Inhibitors

Recently, HIV-1 budding inhibitors that target the interaction between Gag p6 and TSG101 were identified. Using SICCLOPS / RTHS system (split intein-mediated circular ligation of peptides and proteins/ reverse two-hybrid system), Tavassoli *et al.* (2008) identified a cyclic peptide, Tat 11 (IYWNVSGW) that disrupted Gag p6-TSG101 and HRS-TSG101 interactions at the same degree and inhibited VLP release. However, it was not shown yet whether or not Tat 11 cyclic peptide can inhibit HIV-1 replication. Another budding inhibitor targeting Gag p6-TSG101 identified so far is the HIV-1 p6 nonapeptide, also known as Gag PTAP peptide (PEPTAPPEE), which is derived from Gag p6 amino acid sequence (Im *et al*., 2010). However, the binding affinity (K_d) of this peptide is greater than 50 μM, which is outside the useful range (Kim *et al*., 2011). This peptide was modified and

developed to obtain higher binding affinity to TSG101 (Kim *et al*., 2011 and Liu *et al.,* 2010) but the ability of these peptides to inhibit HIV-1 release remains to be tested.

Aim of the Study

As a continued quest for a curative and more potent HIV-1 therapy, this study aimed to identify HIV-1 replication inhibitors using Gag-TSG101 interaction as a target by an ELISA-based high throughput screening system of a core chemical library containing 9,600 small molecules with diverse chemical structures and by further screening through cytotoxicity assay and HIV-1 infection assays. This study also aimed to characterize the mechanisms of action of the hit Gag-TSG101 inhibitors having antiretroviral activity based on its effect on Gag-TSG101 interaction *in vitro* and its effect on Gag VLP release. Moreover, this study further aimed to determine the binding partner protein of the hit small molecules to exert its inhibitory effect as well as to determine their possible docking sites on the binding partner protein to gain more insights on their mechanisms of action.

Materials and Methods

Small molecules and peptide

Nine thousand six hundred small molecules were obtained from Drug Discovery Initiative, The University of Tokyo. Stock solution of each small molecule (10 mM) was prepared in dimethyl sulfoxide (DMSO, Nacalai Tesque, Kyoto, Japan).

PEPTAPPEE peptide (SCRUM, Kashiwa, Japan) stock solution (10 mM and 100 mM) was prepared in distilled water. Both stock solutions of small molecules and peptides were stored at -20°C until needed.

Cell cultures and transfection

HeLa cells and human embryonic kidney 293T, HEK293T cells were maintained in Dulbecco's Modified Eagle's Medium (Invitrogen, Carlsbad, CA) while CEM cells were grown in RPMI. Both media were added with Pen Strep Glutamine (PSG, GIBCO Industries Inc., Los Angeles, CA) and 10% fetal bovine serum (Sigma, St. Louis, MO). All cells were cultured at 37°C in 5% CO_2. All transfection experiments were performed using FuGENE HD (Roche Diagnostics, Basel, Switzerland).

Construction of expression plasmids

The pET28a bacterial expression vector encoding His-Tsg101 UEV was constructed as follows: the cDNA from HeLa cells, which corresponds to Tsg101 UEV was amplified by PCR and cloned into pET28a within restriction enzyme sites, *BamH*I and *Not*I. Primers used Tsg101 sequencing are as follows; Tsg101BamHI F (5'AAAGGATCCGCGGTGTCGGAGAGC 3') and Tsg101 Xho R2 (5' AAACTCGAGTCACTACCCCGTTGCCTGGTATGG 3'). pGEX-6P-3/GST-Gag,

pCAGGS/Gag, pCAGGS/HA-Gag and pCAGGS/FLAG-TSG101 plasmids were provided by Dr. Nopporn Chutiwitoonchai (Viral Infectious Diseases Unit, RIKEN). In addition, the infectious molecular clone HIV-I NL43-2 was described previously (Adachi *et al*., 1986).

Expression and purification of proteins

The GST/pGEX-6P-3, GST-Gag/pGEX-6P-3 and His-TSG101 UEV/pET28a plasmid constructs were transformed into *E.coli* BL21. GST and GST-Gag protein were purified using Glutathione Sepharose Fast Flow beads while His-TSG101 UEV/pET28a was purified using His Gravitrap affinity column (GE Healthcare, Buckinghamshire, UK). GST, GST-Gag and His-TSG101 UEV purified proteins were filtered using Amicon filter device. HA-Gag and Flag-TSG101 were purified from transfected 293T cells using HA beads (Sigma) and Flag M2 beads (Sigma). Final protein purification step was performed by incubation of beads with HA peptide and Flag peptide, respectively.

Enzyme-linked immunosorbent assay (ELISA)-based binding assay

The wells of 96-well microplates were coated with Glutathione S-Transferase (GST) or GST-Gag (10 μg/ml) for 16 h at 4°C. After washing for ten times, the wells were incubated with phosphate buffered saline (PBS) containing 0.05% Tween 20 and 2% bovine serum albumin (BSA, Chon Fraction V powder, IWAI, Tokyo, Japan) for 2 h at room temperature. The wells were washed and added with His-TSG101 UEV (2 μg/ml) together with HIV-1 Gag peptide PEPTAPPEE (SCRUM,) or test compounds and incubated for 2 h at 4°C. After washing step, the horseradish peroxidase (HRP)-conjugated anti-His tag monoclonal antibody (mAb) (MBL, Nagoya Japan) was added and incubated for 2 h at room temperature. Following the washing step, microplates were added with tetramethybenzidine (TMB, Thermo Scientific, Rockford, IL). After incubation at room temperature for 30 min, the

amount of surface bound TSG101 was estimated by measuring the optical density of the wells at 450nm using an ELISA plate reader (Wallac ARVO^{TM} SX 1420; Perkin Elmer, Waltham, MA).

Analyses of the parameters of HTS assay

The quality of each ELISA-binding assay performed during the development of high throughput screening system (HTS) and the actual ELISA-binding assays performed during screening were assessed by three statistical parameters: signal to noise ratio (S/N), signal to background ratio (S/B). S/N measures the degree of confidence with which the signal can be regarded as real (Zhang *et al*., 1999) and is defined by:

$$S/N=\frac{\text{Average}_{\text{negative control (GST-Gag)}}-\text{Average}_{\text{positive control (GST)}}}{\text{SD}_{\text{positive control (GST)}}}$$

S/B signal is defined by:

$$S/B=\frac{\text{Average}_{\text{positive control (GST)}}}{\text{Average}_{\text{negative control (GST-Gag)}}}$$

Z'-factor is a statistical coefficient reflective of both the assay dynamic range and the data variation associated with the signal measurements, making it a suitable parameter to assess the quality of the assay (Zhang *et al*., 1999). It is defined by:

$$Z' = 1-\frac{3\text{xSD}_{\text{positive control (GST)}}+3\text{xSD}_{\text{negative control (GST-Gag)}}}{\text{Average}_{\text{positive control (GST)}}-\text{Average}_{\text{negative control (GST-Gag)}}}$$

Cell proliferation assay using water-soluble tetrazolium salt-1 (WST-1) assay

The effect of small molecule on cell viability at varying concentrations was determined by WST-1 assay (TaKaRa Bio Inc., Shiga, Japan). Human CD4+ T cell line, CEM cells (1 x 10^3 cells / well) treated with DMSO or with varying concentrations of the small molecule were seeded in 96-well microplate. DMSO treated and untreated CEM cells were used as controls. After 96h incubation at 37°C with 5 % $C0_2$, 10 µl of WST-1 were added into the wells and incubated for 30 min at 37°C. Absorbance at 450 and 690 nm were measured and the percentage of cell viability in each well was calculated based on the absorbance values of the controls. The 50% cytotoxic concentration (CC50) of each candidate small molecule was calculated in comparison with the viability of DMSO-treated cells.

Generation of virus stocks

HIV-1 NL43-2 viruses were propagated from 293T cells at 37°C for 48 h in 5% CO_2. Initially, 293T cells (2.5 x 10^6 cells) were seeded in Dulbecco's Modified Eagle's Medium (Invitrogen) with Pen Strep Glutamine (PSG, GIBCO Industries Inc., Los Angeles, CA) and 10% fetal bovine serum (Sigma) in 10cm NUNC dish (Thermo Scientific) and grown overnight at 37°C in 5% CO_2. A 10 µg HIV-1 pNL43-2 plasmid was mixed with Opti-MEM, reduced serum medium (Thermo Scientific,) and FuGene HD (Roche Diagnostics, Basel, Switzerland) in a 1:50:3 ratio. Cells were then transfected with the plasmid mixture in a fresh medium and continued to grow at 37°C for 48 h in 5% CO_2. Titers of virus stocks were measured based on the amount of p24 antigen in each culture supernatant by a HIVp24 enzyme-linked immunosorbent assay (ELISA) kit (MBL) using p24 mAb (MBL).

HIV-1 infection assay

CEM cells (2.5 x 10^4 cells/well) were infected with HIV-1 NL43-2 virus at 20 ng by spinoculation at 25°C for 1 h at 1,200 x g as described earlier (O'Doherty *et al*., 2000). Cells were suspended in RPMI 1640 containing 10% Fetal Bovine Serum (FBS, Thermo Scientific) and PSG. Uninfected or infected cells were then treated with small molecule at varying concentration and transferred into 24-well plate. The plates were incubated for up to 4 days post infection and a small amount of the culture supernatant was collected. Virus production was assessed by p24 ELISA (Ryukyu Immunology Corporation, Japan) using the culture supernatant on day 4 post infection. The 50% infectivity concentration (IC50) of each candidate small molecule was calculated after 4 days of infection.

GST pull-down assay

Glutathione Sepharose beads coupled with GST or GST-tagged Gag (20 μl) was incubated with purified Flag-tagged TSG101 (2 μg) using end to end rotation at 4°C overnight in the presence or absence of small molecule at different concentrations (1, 5 and 10 μM). After washing the beads with Net buffer (10 mM Tris pH 7.8, 150 mM NaCl and 0.05% NP-40) for five times, the beads were resuspended in SDS sample buffer (50mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 12.5mM EDTA and 0.02% bromophenol blue) and the bound fractions were analyzed Western blotting with TSG101 mAb (Abcam, Cambridge, UK).

Photo-cross-linked small molecule affinity beads assay

The candidate small molecules, HSM-9 and HSM-10 were cross-linked into Sepharose beads as previously described (Kanoh *et al*., 2005, Kanoh *et al*., 2006). Briefly, N-hydroxysuccinimide-activated beads were washed three times with 1mM aqueous HCl and

with coupling solution (0.1M $NaHCO_3$ and 50% dioxane mixture). Beads were added with a solution of photoaffinity linker diluted in coupling solution and incubated at 37°C for 2 h on a rotor. It is then washed with coupling solution for five times and blocked with 1 M ethanolamine in 0.1M Tris HCl (pH 8.0) buffer at 37°C for 1h on a rotor. Beads were then washed three times with Milli-Q water and methanol in a spin column and transferred to a glass sample vial. A methanol solution containing each of the candidate small molecules and the mixture was concentrated and dried *in vacuo*. The beads with the small molecule were irradiated at 365nm ($4J/cm^2$) and washed with methanol to yield the HSM-9/HSM-10 cross-linked affinity beads.

Purified HA-Gag (100 ng) or His-TSG101 (100 ng) was incubated with the HSM-9 and HSM-10-cross-linked affinity beads or uncross-linked beads at 4°C for 24 h. The beads were then washed five times with wash buffer, which contains 10mM Tris (pH7.8), 150 mM NaCl and 0.05% NP-40. The protein that bound to the HSM-9 and HSM-10 small molecules was then separated in 10% SDS-PAGE gels. Protein detection was performed by western blot analysis using anti-Gag polyclonal antibody (pAb; Bio Academia) and anti-TSG101 mAb (Abcam).

In silico analyses of the docking sites of HSM-9 and HSM-10 in HIV-1 Gag p6

In silico analysis of the binding sites of HSM-9 and HSM-10 small molecules in HIV-1 Gag p6 were performed using Molecular Operating Environment (MOE Software; Chemical Computing Group). HSM-9 and HSM-10 structures with stable conformations were constructed and identified using MOE. For the purpose of comparison, stable conformations of the other 4 candidate small molecules with no antiretroviral activity (HSM-1, HSM-2, HSM-4 and HSM-7) were also constructed. Solution structure of HIV-1 p6 protein available at the Protein Data Bank (PDB) with PDB ID 2c55 (Fossen *et al*., 2005) was selected and its

amino acid sequences were compared with the HIV-1 NL4-3 strain (Gen Bank: AAA44987, Adachi *et al*., 1986). HIV-1 p6 structure with stable conformation was then prepared and the possible binding sites in HIV-1 p6 were identified. Docking simulation analyses were then performed. Interaction between HIV-1 p6 and each small molecule were evaluated based on docking scores (U-dock).

Gag virus-like particle (VLP) assay

A Gag expression vector, pCAGGS/Gag (0.5 μg-2 μg) was transfected into 293T cells (1.5-3.0 x 10^5 cells/well) in a 12 well or 24-well plate NUNC Multidish (Thermo Scientific)\. After 24-h incubation, culture supernatant was collected. By ultracentrifugation at 40,000 rpm for 40 min at 4°C using Beckman SW50 (Beckman Coulter Inc., Brea, CA), the VLP in the culture supernatant were isolated through a 20% sucrose cushion and PBS buffer. The VLPs in the pellets were suspended in SDS sample buffer and analyzed by SDS-PAGE and Western blotting with anti-Gag pAb (Bio Academia, Osaka, Japan) and anti-β-actin mouse mAb (Sigma).

Western blotting

For the analysis of the cell-associated proteins, cells were lysed with Net buffer with cOmplete EDTA-free protease inhibitor cocktail (Roche Life Science) and the total protein concentration was determined by BCA assay (Thermo Scientific) using a BSA standard (Thermo Scientific). Proteins samples were boiled at 100 °C for 5 min and separated by sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) electrophoresis in 10% - 15% SDS-PAGE gel. Gels were transferred on Immobilon-P membrane polyvinyldene difluoride (PVDF) membrane (Merck, Darmstadt, Germany). The following antibodies were used anti-Gag rabbit pAb (Bio Academia), anti-FLAG M2 mouse mAb (Sigma), anti-GST rabbit pAb

(MBL), anti-β-actin mouse mAb (Sigma), HRP-conjugated goat anti-rabbit IgG (Amersham Biosciences, Buckinghamshire, UK). Development of the chemiluminiscent signal was performed using SuperSignal West Pico chemiluminiscent substrate (Thermo Scientific) and protein bands were visualized using an AlphaImager 3400 imaging system (Alpha Innotech Corparation, San Leandro, CA) or Hyperfilm MP (Amersham Biosciences,) with Hi-RENDOL/Hi-RENFIX (Fujifilm, Tokyo, Japan)

Results

Development of a new HTS system using (ELISA)-based binding assay

To search for small molecules inhibiting HIV-1 replication, I developed a new ELISA-based binding assay to identify the small molecule inhibitors of Gag-TSG101 interaction (Figure 5).

Firstly, recombinant bacterial vectors that express either the full-length Gag protein or the TSG101 protein with UEV domain were constructed. Gag was inserted into the bacterial expression vector, pGEX-6P-3 containing an N-terminal GST tag while TSG101 UEV was inserted into pET28a bacterial expression vector containing an N-terminal His tag. GST-Gag (80 kDa) and His TSG-101 UEV (18 kDa) proteins were purified from BL21 *E.coli* using Glutathione Sepharose beads and His-affinity column, respectively (Figure 5A). As a negative control, GST protein (27 kDa) was purified from pGEX-6P-3 transformed BL21 *E.coli* using Glutathione Sepharose beads as well (Figure 5A). Band sizes of each protein correspond to its expected size as shown in CBB staining (Figure 5A).

Next, ELISA-based binding assay was developed by determining the optimal conditions of the assay as well as the optimal concentrations of the purified proteins (Figure 5B). In this assay, purified GST (positive control) or GST-tagged Gag protein was immobilized on the 96-well microplate. Following the washing and blocking steps, His-TSG101 UEV protein was added and incubated with DMSO (negative control) or with the small molecules. Gag-TSG101 interaction was detected by anti-His HRP monoclonal antibody and addition of TMB substrate. The strength of binding between the two proteins were measured based on absorbance values.

Moreover, to assess the quality of ELISA-based screening assay, the absorbance values of the positive and negative controls (Figure 5C) were compared and each assay was validated

based on the Z' factor, a coefficient which reflects both the assay signal dynamic range and the data variation associated with the signal measurements (Zhang *et al*., 1999). Using these parameters, it was observed that for every plate assay performed, Z' factor was within the required range, that is greater than 0.5 but not higher than 1.0. A representative of the assay showed that the Z' factor was 0.76 and S/B ratio was 21.9 (Figure 5C and 5D).

Thus, this assay appeared to be good system to identify the small molecule inhibitors of Gag-TSG101 interaction.

Screening of small molecule inhibitors of viral Gag-host TSG101 protein interaction

A core library containing 9,600 diverse small molecules was screened by ELISA-based binding assay to identify the small molecule inhibitors of Gag-TSG101 interaction. Absorbance value from each small molecule were normalized as percentage of inhibition on Gag-TSG101 binding relative to the average absorbance values of positive and negative controls of each assay. To identify the primary hit small molecules, 3 standard deviations from the mean of all the percentage of inhibition by all small molecules was used, which is at 30%. Among the 9,600 small molecules screened, eighty-one of which inhibited the GST-Gag-His TSG101 UEV interaction (Figure 5E).

To further select the true primary hits, another ELISA-binding assay experiment was performed using only the eighty-one small molecules. Based on the mean from two independent ELISA-binding assay experiments, eleven small molecules were able to inhibit Gag-TSG101 interaction ranging from 30% to 80% binding inhibition (Figure 5F). Small molecules HSM-4, HSM-9 and HSM-10 showed the highest percentage of inhibition at 75.5% to 80%. The chemical structures of the eleven candidate small molecules were varied and no similarity in structure was observed (Figure 6). The molecular weight of each candidate small molecule ranges from 200 to 450.

Effect of small molecules on the cell viability of $CD4^+$ cell line, CEM

Given that the Gag-TSG101 binding is indispensable in HIV-1 life cycle particularly in the viral assembly and release, interference on this binding by the candidate small molecules might block HIV-1 replication. For this reason, the concentration of each candidate small molecule with minimal effect on the cell viability of CEM was determined and the ones with 50% cell viability greater than 40 μM for the HIV-1 infection assay were selected. Cell proliferation in the presence of each candidate small molecules was determined using WST-1 assay. Among the eleven candidate small molecules, which inhibit GST-Gag and His-TSG101 UEV interaction, six of these were observed to have 50% cell viability in CEM (CC50) at concentrations greater than 40 μM (Table 1). The concentrations of each small molecule used in the successive experiments of this study were within the CC50 concentrations of each small molecule.

Effect of small molecules on HIV-1 infection

Several studies have reported that inhibiting Gag-TSG101 interaction affects HIV-1 replication. To test whether the primary hit small molecules that inhibit Gag-TSG101 interaction affect HIV-1 replication, HIV-1 infection assay was performed. CEM cells were infected with HIV-1 NL43 and treated with the serially diluted candidate small molecules. Virus replication was assessed using the supernatants of the culture by p24 ELISA at 4 days post infection. A dose-dependent decrease as well as a 50% decrease in HIV-1 replication was not observed when cells were treated with HSM-1, HSM-2, HSM-4 and HSM-7 at varying concentrations (data not shown) suggesting that these candidate small molecules did not exert inhibitory effects on HIV-1 replication. Interestingly, supernatants from HIV-1 infected cells treated with HSM-9 and HSM-10 showed a decrease in p24 concentration in a dose dependent manner on 4 days post infection (Table 1, Fig. 7A and 7B). Both HSM-9 and

HSM-10 strongly inhibited the viral replication and reduced the p24 value to 50% (IC50) at 1.5 μM ± 0.01and 9.8 μM ± 1.40 μM (Table 1). This suggests that inhibition of Gag-Tsg101 interaction could block HIV-1 replication. Comparing the selective index (SI) between these small molecules, the SI of HSM-9 is about 5 fold higher than that of HSM-10 (Table 1). Taken together, successive screening of 9,600 small molecules revealed that HSM-9 and HSM-10 small molecules, which strongly blocked Gag-TSG101 interaction, also potently inhibited HIV-1 replication (Table 2).

HSM-9 and HSM-10 inhibit Gag-TSG101 interaction

In order to determine whether HSM-9 and HSM-10 inhibit Gag-TSG101 in a dose dependent manner, another ELISA binding assay was performed in the presence of varying concentrations of each candidate small molecule (1, 5 and 10 μM). Both HSM-9 and HSM-10 were able to inhibit the interaction between GST-Gag and His-TSG101 UEV in a dose dependent manner with EC50 6.2 ± 0.88 μM and 7.8 ± 0.28 μM, respectively (Fig. 8A and 8C). In contrast, the inhibition against Gag-TSG101 interaction by HSM-9 and HSM-10 at 10μM during ELISA-based screening assay showed higher percentages of inhibition (~80%) than those of their percentages of inhibition when increasing concentrations were used. These differences in the percentage of inhibition might be accounted by the different batches of proteins used in these two assays. Nonetheless, both results confirm the ability of HSM-9 and HSM-10 to inhibit Gag-TSG101 interaction.

To further confirm the inhibition activity of HSM-9 and HSM-10 against Gag-TSG101 interaction, GST pull down assay was performed. GST or GST-Gag bound to glutathione sepharose beads was incubated with full length FLAG-TSG101 with or without HSM-9 and HSM-10 small molecules at 1, 5 and 10μM. Western blotting analyses showed that the binding of full length FLAG-TSG101 to GST-Gag decreased in a dose-dependent manner in

the presence of HSM-9 and HSM-10 (Figures 8B and 8D). However, Gag-TSG101 binding was more highly inhibited when HSM-9 was added at 10uM (>80%) while when HSM-10 was added at the same concentration, the Gag-TSG101 binding was only inhibited for lesser than 40%. Notwithstanding, this result further confirms the dose-dependent inhibitory activity of both HSM-9 and HSM-10 on Gag-TSG101 binding.

HSM-9 and HSM-10 target HIV-1 Gag, but not TSG101

To determine which of the two interacting proteins do HSM-9 and HSM-10 directly interacted, a photo-cross-linked small molecule affinity beads assay was performed (Figure 9). Candidate small molecules were cross-linked to sepharose beads, added with a photo-cross linker and UV irradiated to yield a photo-cross linked small molecule beads. Beads were incubated with either HA-tagged Gag or FLAG-tagged TSG101 and the proteins bound to the small molecule-linked beads were determined by Western blotting using anti-Gag mAb and anti-TSG101 mAb, respectively. Results from this assay suggest that HA-Gag proteins coprecipitated in the sample containing HSM-9 and HSM-10 affinity linked beads (Figure 9). This indicates that both HSM-9 and HSM-10 small molecules specifically bind to the Gag protein and not to the TSG101.

HSM-9 and HSM-10 have different docking sites in HIV-1 p6

In order to better understand the Gag-dependent inhibitory activity of HSM-9 and HSM-10, possible binding sites of each small molecule in HIV-1 p6 were presumed by *in silico* docking simulation analysis. Solution structure of HIV-1 p6 protein, PDB ID 2c55 (Fossen *et al*., 2005) contains identical amino acid sequences with the HIV-1 NL4-3 strain (Gen Bank: AAA44987, Adachi *et al*. 1986) and thus, it was used to construct the HIV-1 p6 structure with stable conformation (Figure 10A). Five possible docking sites in HIV-1 p6 were

identified and were used in the docking simulation analysis (Figure 10B). Docking simulation analysis reveals that the U-dock score of HSM-10 at site-3 is higher than the other molecule simulations (U-dock: -30.10kcal/mol) (Table 3). The simulation also showed that a hydrogen bond was formed between the HSM-10 hydroxyl group and the alanine of main chain in PTAP sequence (Table 3, Figures 10D). Aside from the alanine in PTAP, docking simulation complex models of HIV-1 p6 protein and HSM-10 also identified a hydrogen bond between the threonine residue of PTAP and HSM-10 (Figure 10D). In contrast, docking simulation complex models of HIV-1 p6 protein and HSM-9 revealed the binding of HSM-9 to the five possible binding sites in HIV-1 p6 but U-dock scores from HSM-9 simulations were lower than HSM-10 simulations and no specific amino acid residue of HIV-1 p6 was able to form a hydrogen bond with HSM-9 (Figure 10C). Collectively, these results suggest that HSM-10 showed a specific binding with the PTAP motif of HIV-1 p6 at alanine and threonine residues, while HSM-9 has at least several non-specific binding sites in HIV-1 p6, which indicate that HSM-10 interacts more strongly with HIV-1 p6 than HSM-9.

HSM-9 and HSM-10 inhibit Gag VLP release

Given that the interaction between HIV-1 Gag and TSG101 plays a major role in HIV-1 assembly and release, the ability of the hit small molecules to inhibit virus release in mammalian cells was investigated by Gag VLP assay. Gag VLP production was measured in the presence or absence of HSM-9 or HSM-10. Human embryonic kidney 293T cells were transfected with untagged full length Gag and incubated with varying concentrations of each candidate small molecules (1, 5 and 10 μM). After 24 h – 48 h transfection, cell lysates and viral-like particles were collected and analyzed by Western blotting. Interestingly, both HSM-9 and HSM-10 efficiently inhibited VLP release in a dose- dependent manner (Figure 11). A significant decrease of Gag VLP expression was distinct in the presence of HSM-9 (1,

5 and 10 μM) at 24 h posttransfection but not after 48h posttransfection. In contrast, a more distinct reduction in VLP production was observed at 48h posttransfection in the presence of HSM-10 but not after 24 h transfection, which showed a slight decrease. Nonetheless, these results indicate that HSM-9- and HSM-10-mediated inhibition on Gag-TSG101 interaction led to interference of VLP/virus release, which presumably account for the decrease in HIV-1 replication.

Discussion

In the present study, two small molecule inhibitors of the viral Gag-host TSG101 interaction were identified through an ELISA-based high throughput screening system. These two small molecules coded as HSM-9 and HSM-10 disrupted Gag-TSG101 interaction, subsequently inhibited Gag VLP/virus release and potently blocked HIV-1 replication. These results indicate that inhibitors of Gag-TSG101 interaction can effectively block HIV-1 replication by inhibiting virus production via specifically binding to viral Gag protein but not to cellular TSG101 protein.

The hit small molecule, HSM-9 inhibited Gag-TSG101 binding *in vitro* in ELISA-binding assay and GST pull-down assay. However, both assays showed a different level of inhibition in the presence of this small molecule. HSM-9 showed a significantly higher inhibition activity on Gag-TSG101 interaction in GST pull-down assay than in ELISA binding assay. It inhibited about 60% of Gag-TSG101 binding at 10 μM in ELISA binding assay when short-length His-TSG101 UEV protein was used. However, this binding was almost completely abolished in the presence of HSM-9 at the same concentration when full-length FLAG-TSG101 protein was used in GST pull-down assay. On the other hand, HSM-10 also inhibited Gag-TSG101 *in vitro* in a dose-dependent manner. Unlike HSM-9, the HSM-10-mediated inhibition on Gag-TSG101 in both protein-binding assays showed an approximately ~50% decrease in Gag-TSG101 binding at 10 μM indicating that the inhibitory activity of HSM-10 on Gag-TSG101 binding is not significantly different when either short length TSG101 protein with UEV domain or full length TSG101 protein is used. These results suggest that aside from TSG101 UEV domain, another site in TSG101, which might have an interaction with the domain/s of Gag, might be directly or indirectly targeted by HSM-9 causing a higher inhibitory activity of HSM-9 than HSM-10 on Gag-TSG101 binding. A

short-length TSG101 UEV protein (~18kDa protein) was used in ELISA binding assay because it was not possible to purify full-length TSG101 from an *E.coli* bacterial expression vector system due to insolubility. A high yield of the TSG101 protein required for a HTS system to screen 9,600 small molecules was attainable only through an *E.coli* bacterial expression system. Collectively, these results suggest a differential inhibitory effect of HSM-9 when either full length or short-length TSG101 is used but inhibition of HSM-10 on Gag-TSG101 was unaffected by this. Notably, a full length GST-Gag was used in both protein-binding assays suggesting that HSM-9-mediated inhibition on Gag-TSG101 binding probably requires the involvement of TSG101 domains or Gag domains other than UEV domain of TSG101 and PTAP motif of Gag to cause a higher inhibitory effect regardless of the target protein of this small molecule.

The fact that HSM-9 and HSM-10 inhibited Gag-TSG101 binding *in vitro*, suggest that these small molecules must bind to either Gag or TSG101 to exert its inhibitory effect. Binding of the hit small molecules to either Gag or TSG101 may denote a difference in their mechanism of actions and inhibitory effects. Photo-cross-linked small molecule affinity beads assay revealed that both HSM-9 and HSM-10 target Gag to exert its inhibitory activities and not to TSG101. Binding to viral Gag and not to TSG101 would denote a lesser risks of cytotoxicity since it might not affect other cellular proteins that normally bind to TSG101.

Screening results suggest that HSM-9 and HSM-10, which inhibited Gag-TSG101 binding, consequently blocked HIV-1 replication in $CD4^+$ T cells, CEM. Thus, the mechanism of blocking HIV-1 replication was explored. Because Gag-TSG101 interaction is essential in the release of new HIV-1 virus particles from the plasma membrane (Garrus *et al*., 2001, Martin-Serrano *et al*., 2001, VerPlank *et al*., 2001), disruption of which might affect the efficiency of the viral particle release. The formation of non-infectious Gag VLP in the presence of the hit

small molecules was assessed based on Gag VLP release assay since Gag protein alone can efficiently form virus-like particles in the absence of the other viral proteins (Gheysen *et al*., 1989). Remarkably, a decrease in VLP/virus production in a dose dependent manner was observed in the presence of HSM-9 at 24 h posttransfection but not at 48 h posttransfection. Likewise, HSM-10 inhibited VLP production in a dose-dependent manner. However, in comparison to HSM-9, its VLP inhibition activity was observed at a later time at 48 h posttransfection. VLP inhibition in the presence of HSM-10 was distinct at 48 h posttransfection. It is reasonable to presume that HSM-9 might be more permeable to cellular membrane or might target many sites in Gag protein and probably disrupted the Gag-TSG101 binding in sufficient amount 24 h posttransfection and subsequently inhibited Gag VLP release. In contrast, HSM-10 might be less permeable or might target specific sites into its target protein, requiring a longer time to reach a quantity sufficient to exert its inhibitory effect on VLP production. However, a cell permeability assay is required to confirm this hypothesis. Moreover, the mechanism of inhibition on VLP/virus production by HSM 9 and HSM-10 differ, but nonetheless these results suggest that interference on the Gag-TSG101 interaction could indeed lead to a reduction in VLP/virus production. These results also reveal that HSM-9 and HSM-10 blocked HIV-1 replication in CEM via inhibition of the virus release.

Although HSM-9 and HSM-10 inhibited Gag-TSG101 interaction *in vitro*; subsequently reduced VLP production; and blocked HIV-1 replication by targeting Gag, these small molecules showed different pattern and degree of inhibition, implying that their mechanisms of action differ, which is presumably dependent on their target sites in HIV-1 Gag.

Several studies reported that besides PTAP motif of p6 region, other regions of Gag might interact with other regions of TSG101 other than the UEV domain. Chamontin *et al*., 2014, observed a defect in TSG101 packaging into NC ΔZF2 particles, indicating that TSG101

could also bind other Gag regions. Remarkably, their results further revealed that NC domain of Gag provides a TSG101 binding site. Compared to Gag p6 protein alone, Gag protein with NC and p6 domains showed higher binding efficiency to TSG101, indicating that Gag-TSG101 binding reaches its optimal level when both NC and p6 domains are present.

The small molecules might also target ubiquitination, a step necessary for HIV budding. An *in vitro* study observed an enhanced affinity of TSG101 to Gag when p6 is expressed with ubiquitin suggesting that ubiquitin modification of HIV-p6 enhances TSG101 binding (Garrus *et al.,* 2001). Moreover, it was also observed that other than PTAP motif of p6, Gag could bind to TSG101 through the ubiquitin moieties attached to several domains of Gag (Freed and Mouland 2006; Gottwein and Krausslich 2005). Thus, aside from the PTAP binding site of TSG101, it is possible that HSM-9 also binds to another site of Gag, which is important to enhance the binding of Gag to TSG101. If this holds true, it led to a higher inhibitory effect of HSM-9 than those of HSM-10. Given that Gag-TSG101 binding is enhanced when ubiquitin moiety or other domains of Gag are present, it is highly possible that one of these small molecules bind to sites in HIV-1 Gag other than p6 and increase the inhibition activity of the small molecules.

Docking simulation analyses revealed that indeed one of these hit small molecules, in this case, the HSM-9 has at least five possible binding sites in HIV-1 p6 while HSM-10 have specific docking sites presumably at alanine and threonine residues of PTAP motif of HIV-1 p6. Although, these simulation data suggest that HSM-10 might interact with HIV-1 p6 more strongly than HSM-9, HSM-10 did not show higher inhibition activity than HSM-9 on Gag-TSG101 binding in GST-pull down assay. Instead, HSM-9 showed higher percentage of inhibition on Gag-TSG101 binding and VLP production. Possible binding of HSM-9 to several binding sites in HIV-1 p6 and possibly to Gag domains other than p6, seemed to enhance inhibition activity of HSM-9 against Gag-TSG101 binding and virus release.

In addition, the docking sites of HSM-9 and HSM-10 might also have an effect on its inhibitory activity on HIV-1 virus release. Several studies demonstrated that besides p6 domain, other domains of HIV-1 Gag are involved in the efficient viral particle production. Jalaguier *et al*., 2011 observed that N-terminal capsid domain is indispensable for an efficient production of HIV-1 VLP. Interestingly, another domain of Gag, the nucleocapsid were observed to function in collaboration with the PTAP motif of p6 domain in the recruitment of cellular machinery, particularly, the TSG101 to facilitate HIV-1 release (Dussupt *et al*., 2009). Incidentally, these findings correlate with the previous studies showing that the role of PTAP within HIV-1 or within heterologous retroviral Gag is dependent on its location within the Gag polyprotein. This suggests that PTAP might require other regions in Gag to facilitate viral budding and release (Parent *et al.,* 1995, Yuan *et al*., 2000 and Dussupt *et al*., 2009). It was observed that mutations or deletions of basic residues in NC domain of Gag severely inhibited virus release. These NC-mutated Gag proteins contain an intact L domain and are able to assemble particles, suggesting an inter-dependent function between NC and PTAP motif to facilitate virus release (Dussupt *et al*., 2009).

Collectively, HSM-9 and HSM-10 small molecules inhibited Gag-TSG101 binding *in vitro* and consequently blocked HIV-1 replication in CEM cells via reduction of VLP/virus production. These small molecules target viral Gag protein to exert its inhibitory effects. Results suggest that HSM-10 might bind specifically to the target site the PTAP motif of Gag p6 but HSM-9 binds possibly to at least five binding sites in Gag p6 and presumably to other Gag domains. Although both small molecules showed inhibition activity against the target viral-host protein interaction and also showed antiretroviral activity, in comparison to HSM-10, possible binding of HSM-9 to sites other PTAP motif of Gag seemed to increase its inhibitory effects on Gag-TSG101 binding and subsequently on virus release and most importantly on HIV-1 replication. Nonetheless, both HSM-9 and HSM-10 show inhibitory

effects against Gag-TSG101 interaction and thus, identified in this study as Gag-TSG101 inhibitors, which have the potential to be developed into novel HIV-1 inhibitors.

As of to date, there are no approved antiretroviral drugs that target the Gag-TSG101 interaction. Tavassoli *et al*., (2008) identified a cyclic peptide that inhibits Gag-TSG101 interaction and subsequently affects VLP production. However, it is not known yet whether this peptide could potently block HIV-1 replication. Thus, this study is the first report of small molecule inhibitors of Gag-TSG101 binding, which subsequently block VLP/virus production, and in turn potently block HIV-1 replication. This study also provides insights on the inhibition activity and mechanisms of action of small molecules inhibitors targeting Gag-TSG101 interaction.

Analysis of the TSG101 UEV-PTAP peptide complex structure revealed that each PTAP residue makes important contacts and the Ala-Pro dipeptide binds in a deep pocket in UEV domain (Pornillos *et al*., 2002a). The PT/SAP motif of Gag p6, which is the possible target of HSM-9 and HSM-10 as suggested by *in silico* analysis, is highly conserved within all HIV-1 subtypes, wherein PTAP motif is more dominant than PSAP. On the other hand, within the retroviruses, the PT/SAP is also highly conserved but PSAP is more dominant than PTAP. Both PSAP and PTAP sequences were observed to bind TSG101 UEV equally well (Pornillos *et al*., 2002a). Thus, these inhibitors maybe used against all HIV-1 subtypes as well as against other retroviruses. However, one possible problem that needs to be addressed is the ability of the small molecules to distinguish PSAP motif of the virus from the PSAP motif of the host since PSAP is also highly conserve in the host cells. In healthy cells, TSG101 normally interacts with endosomal protein, Hrs (hepatocyte growth factor regulated-tyrosine kinase substrate) to facilitate the release of vesicles from endosomes. The binding requires the PSAP motif of Hrs, the same motif used by HIV-1 Gag p6 to bind to TSG101 (Pornillos *et al*., 2003). Thus, it is crucial to determine whether or not the Gag-TSG101

inhibitors identified in this study could distinguish the viral PSAP motif from cellular PSAP motif. Moreover, these small molecule inhibitors should also be assessed against a broad range of clinical and laboratory HIV-1 isolates and subtypes as well as on their ability to specifically recognize viral PT/SAP motif.

Although some aspects and characteristics of these hit small molecules remain elusive, it would be interesting to investigate the potential of these small molecules as anti-retroviral drugs. Results of the study suggest that HSM-9 and HSM-10 have the potential to be used in preclinical and clinical trials since both have the ability to potently block HIV-1 replication by inhibiting Gag-TSG101 interaction and could subsequently reduce the virus production. Given that these inhibitors have different mechanisms of action in comparison to the current antiretroviral drugs, that is through targeting Gag-TSG101 interaction and blocking the virus release, these inhibitors could be developed into a new class of anti-HIV-1 drug. With the current antiretroviral therapy HAART, there is a high risk of viral resistance but this could be overcome if HAART is combined with a new class of HIV-1 drug having novel mechanisms of action such as the inhibitors identified in this study. However, optimization of these Gag-TSG101 inhibitors is necessary to resolve the cytotoxicity issues and to increase the antiretroviral activity. Optimization could be attained by modification of structures of HSM-9 and HSM-10 that could resolve the cytotoxicity problem and increase the antiretroviral activity as well as improve the absorption of the compound while retaining its ability to potently inhibit Gag-TSG101 and to reduce virus production. Identification of the best-modified structures of the small molecules with higher potency could lead to the approval of HSM-9 and HSM-10 as preclinical candidate drugs against HIV-1.

Conclusion

Collectively, the results presented herein suggest that HSM-9 and HSM-10 inhibit Gag-TSG101 interaction via binding to Gag, which in turn reduces virus production, leading to a block in HIV-1 replication. In other words, HSM-9 and HSM-10 inhibited HIV-1 replication through reducing VLP/virus production. The results of this study demonstrate that viral Gag-host TSG101 interaction is a valuable target for inhibiting HIV-1 replication and that drugs interfering Gag-TSG101 interaction may not only be beneficial against HIV-1, but also against other viruses that require Gag-TSG101 binding to facilitate efficient viral budding and release.

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Figures and Tables

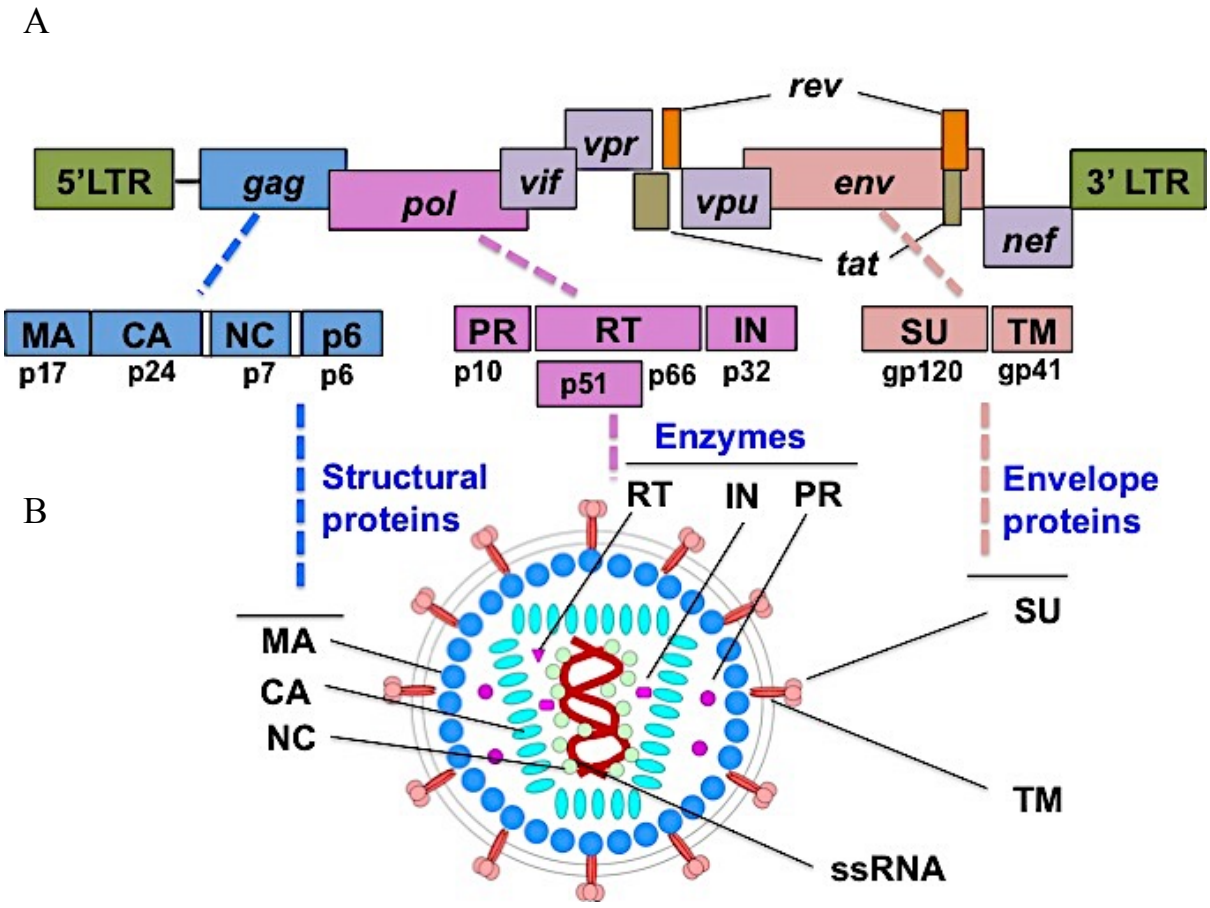


Figure 1. HIV-1 genome organization and mature HIV-1 virion. **(A)** HIV-1 genome consists of three major genes; *gag, pol* and *env*, which encode for structural proteins, viral enzymes and coat proteins of the virion. It is flanked by two LTR (long-terminal repeats) at 5' and 3' regions and contains four accessory genes namely, *vif, vpr, vpu* and *nef* as well as regulatory genes, *rev* and *tat*. (B) Schematic representation of a mature HIV-1 virion. Mature HIV-1 virion is composed of individual proteins cleaved from Gag, Env and Pol precursor polyproteins. TM (transmembrane) protein protrudes through the membrane and its external portion is bound to surface protein (SU). Both constitute the envelope of the virion. Matrix (MA), which lies underneath the lipid membrane, forms the outer shell. Farther inside the

virion, capsid (CA) forms a conical shape enclosing the core containing the 2 strands of single stranded RNA (ssRNA), which is in complex with nucleocapsid (NC). This complex is associated with reverse transcriptase (RT) and integrase (IN) molecules while protease (PR) molecules are located outside the core.

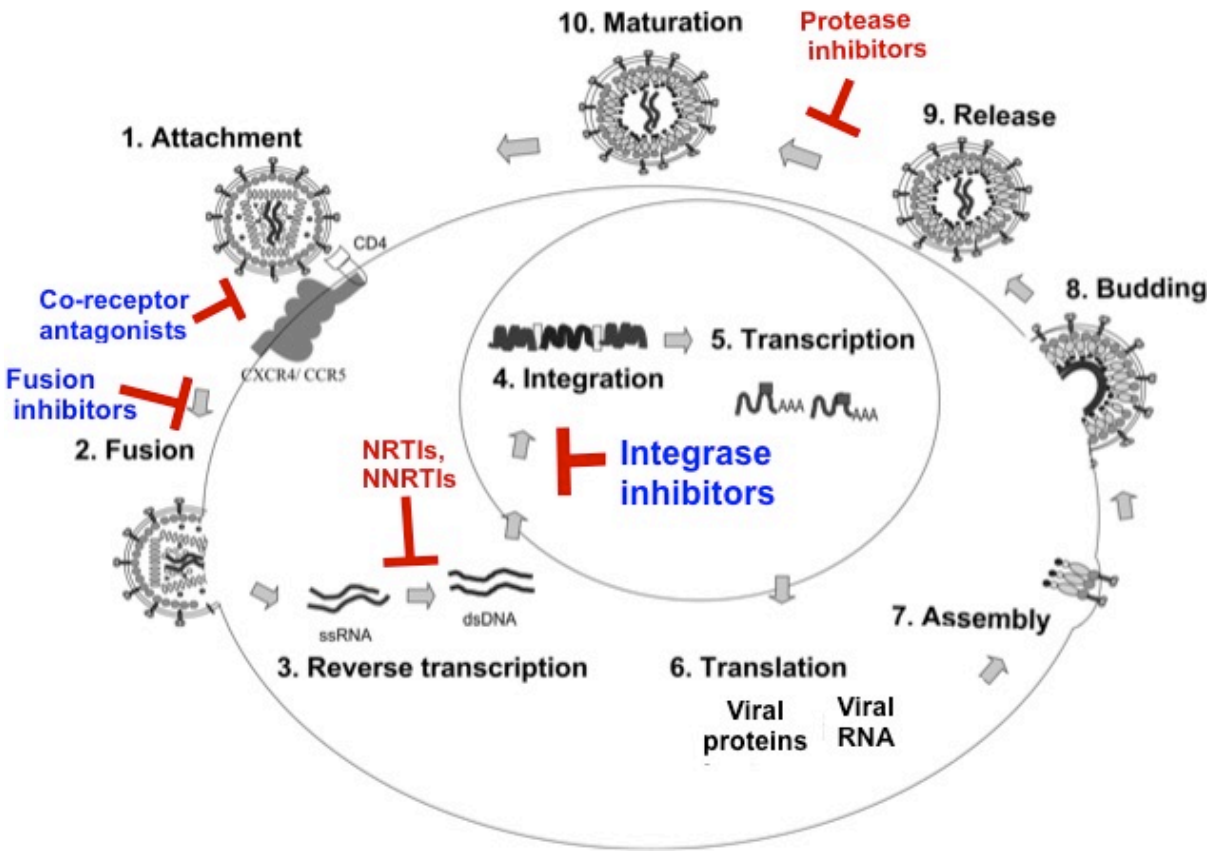


Figure 2. HIV-1 replication cycle. A mature HIV-1 virion enters its target cell by (1) attachment to the CD4 receptor and its co-receptors, CXCR4 or CCR5. (2) Fusion of viral membrane and host membrane then occurs, leading to the uncoating of the viral proteins and ssRNA in the cytoplasm. By (3) reverse transcription, viral ssRNA is transcribed into dsDNA.Viral dsDNA are then (4) integrated into the host's chromosomal DNA. (5) By transcription, new viral RNA are synthesized and exported from the nucleus to the cytoplasm.(6) These are translated into precursor polyproteins and viral genome RNAand are targeted to the plasma membrane for (7) virion assembly and (8) budding. An immature progeny virion is released from the infected cell (9) and (10) matures by proteolytic cleavage. Current antiretroviral drugs, which target each step of HIV-1 replication cycle are indicated. Nucleoside reverse transcriptase inhibitors (NRTIs); non-nucleoside reverse transcriptase inhibitors (NNRTIs); integrase strand transfer inhibitors (InSTIs); and allosteric integrase inhibitors (ALLINIs).

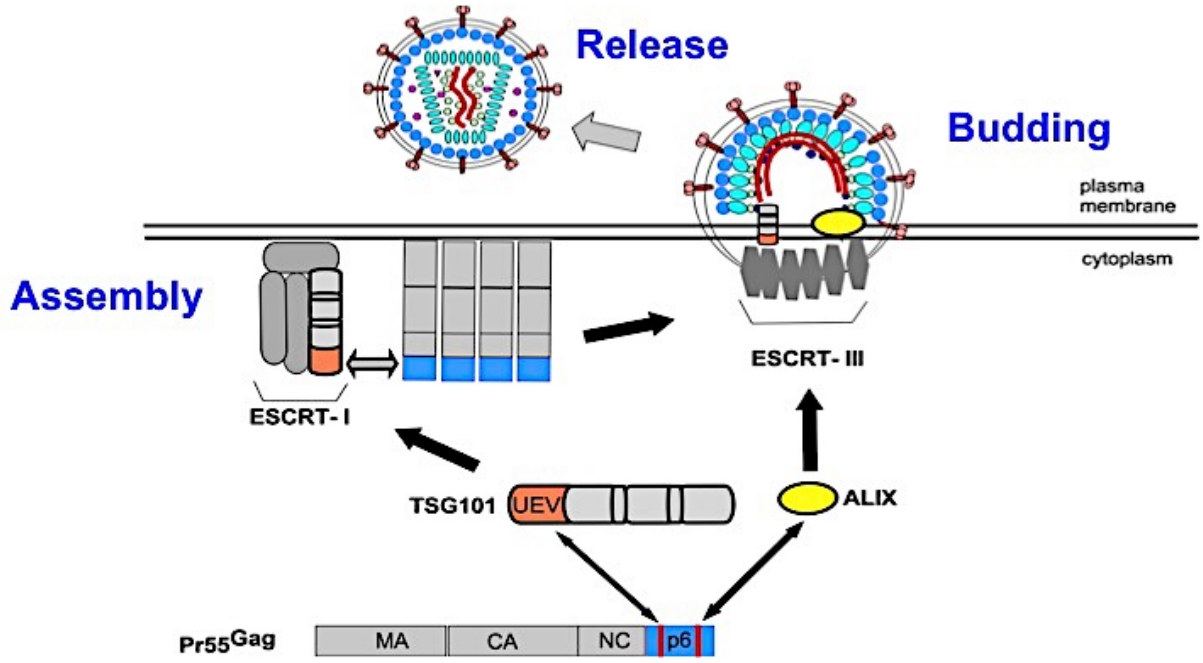


Figure 3. The late phase of HIV-1 replication: HIV-1 assembly, budding and release. During viral assembly, HIV-1 Gag p6 directly interacts with ESCRT-I subunit, TSG101 to takeover the cellular ESCRT machinery for the budding and release of new virions. TSG101 and along with ESCRT-I complex is recruited at the plasma membrane by HIV-1 Gag. At the budding stage, Gag proteins interact with other ESCRT subunits (e.g. ALIX), leading to the formation of ESCRT-III complex, which recruits other cellular factors (e.g. VPS4) to dissociate the membrane-bound ESCRT complexes and to provide energy for membrane fission. New immature virions are released from the plasma membrane.

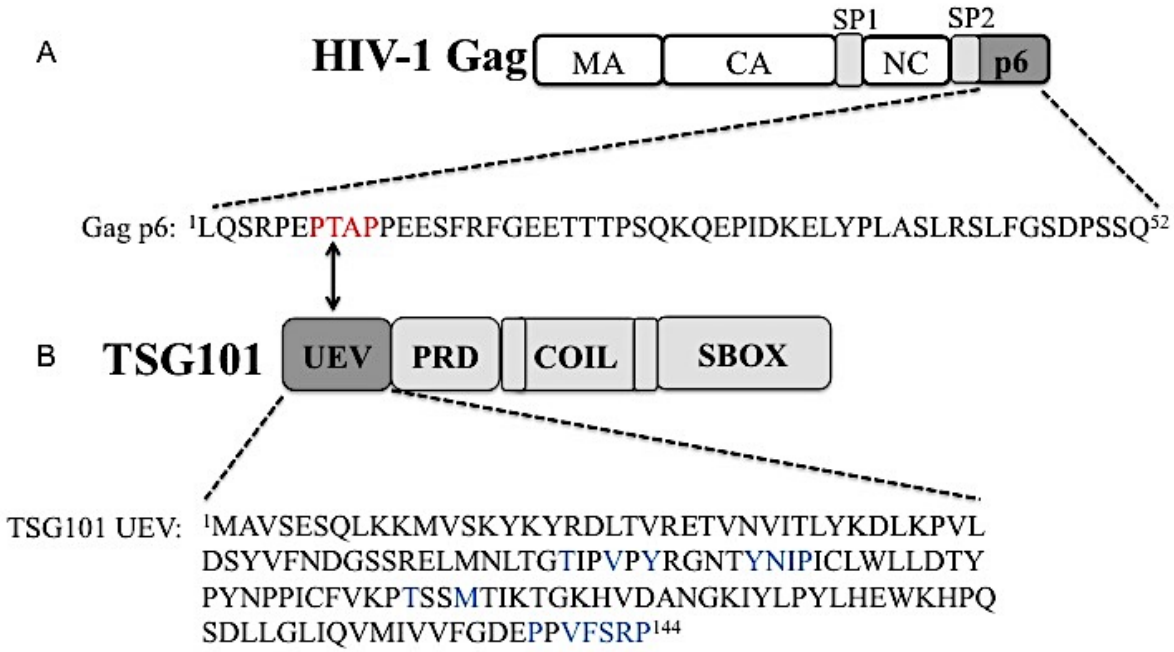
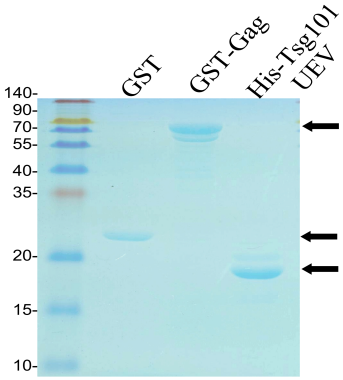


Figure 4. Domain organization of HIV-1 Gag and host protein TSG101. (A) Schematic representation showing the domain organization of HIV-1 Gag precursor polyprotein, $Pr55^{Gag}$. HIV-1 Gag is consists of MA, CA, NC and p6 domains as well as 2 spacer peptides, SP1 and SP2. The p6 domain contains the binding motif for TSG101 (PTAP; red). (B) Schematic showing the domain organization of TSG101. TSG101 consists of four domains: UEV, PRD, COIL, and SBOX. TSG101 UEV domain contains the binding PTAP motif of Gag p6 domain and UEV residues that are important for this binding are shown in blue.

A



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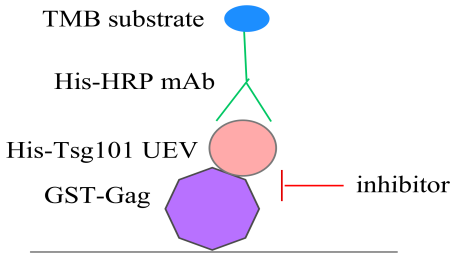

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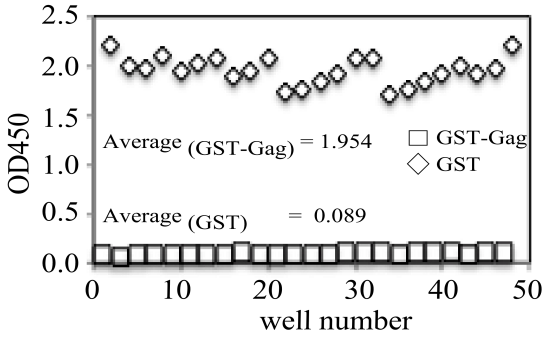


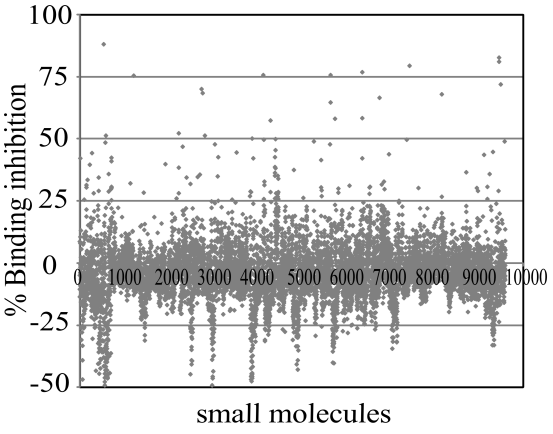
D

$$S/N = \frac{\text{Average}_{(GST\text{-}Gag)} - \text{Average}_{(GST)}}{\text{SD}_{(GST)}} = 144.7$$

$$S/B = \frac{\text{Average}_{(GST)}}{\text{Average}_{(GST\text{-}Gag)}} = 21.9$$

$$Z' \text{ factor} = 1 - \frac{3 \times \text{SD}_{(GST)} + 3 \times \text{SD}_{(GST\text{-}Gag)}}{\text{Average}_{(GST)} - \text{Average}_{(GST\text{-}Gag)}} = 0.76$$

E



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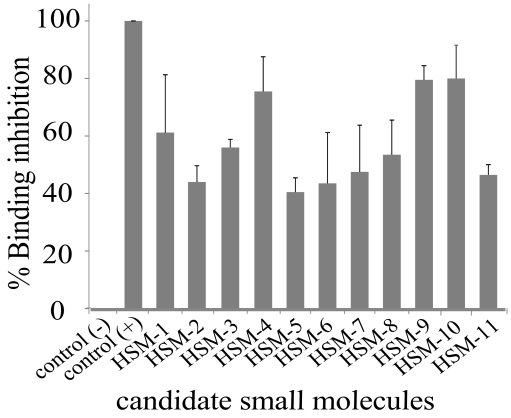


F**igure 5. Development of a new HTS screening system using ELISA-based binding assay and result of its screening.** A) Purified proteins used in ELISA-based binding assay: GST=27kDa; GST-tagged Gag (GST-Gag) = 80kDa; and His-tagged TSG101 UEV (His-TSG101 UEV) =18kDa. B) Schematic representation of ELISA-based binding assay. Purified GST-Gag is immobilized on the 96-well microplate and incubated with His-Tsg101 UEV in the presence of DMSO or small molecule. Bound His-TSG101 to GST-Gag was detected using HRP-conjugated anti-His MAb. C) Determination of parameters of the screening assay targeting the interaction between His-TSG101 and GST-Gag. GST was used a negative control. D) To validate the constructed system for HTS assays, the single-to-noise ratio (S/N), single-to-background (S/B) ratio and Z' factor were calculated. Two independent experiments were performed, and one representative result is shown. E) Result of the ELISA-based binding assay screening of 9,600 small molecules. F) Percent of Gag-TSG101 binding inhibition by the 11 primary hit small molecules from two independent ELISA experiments.

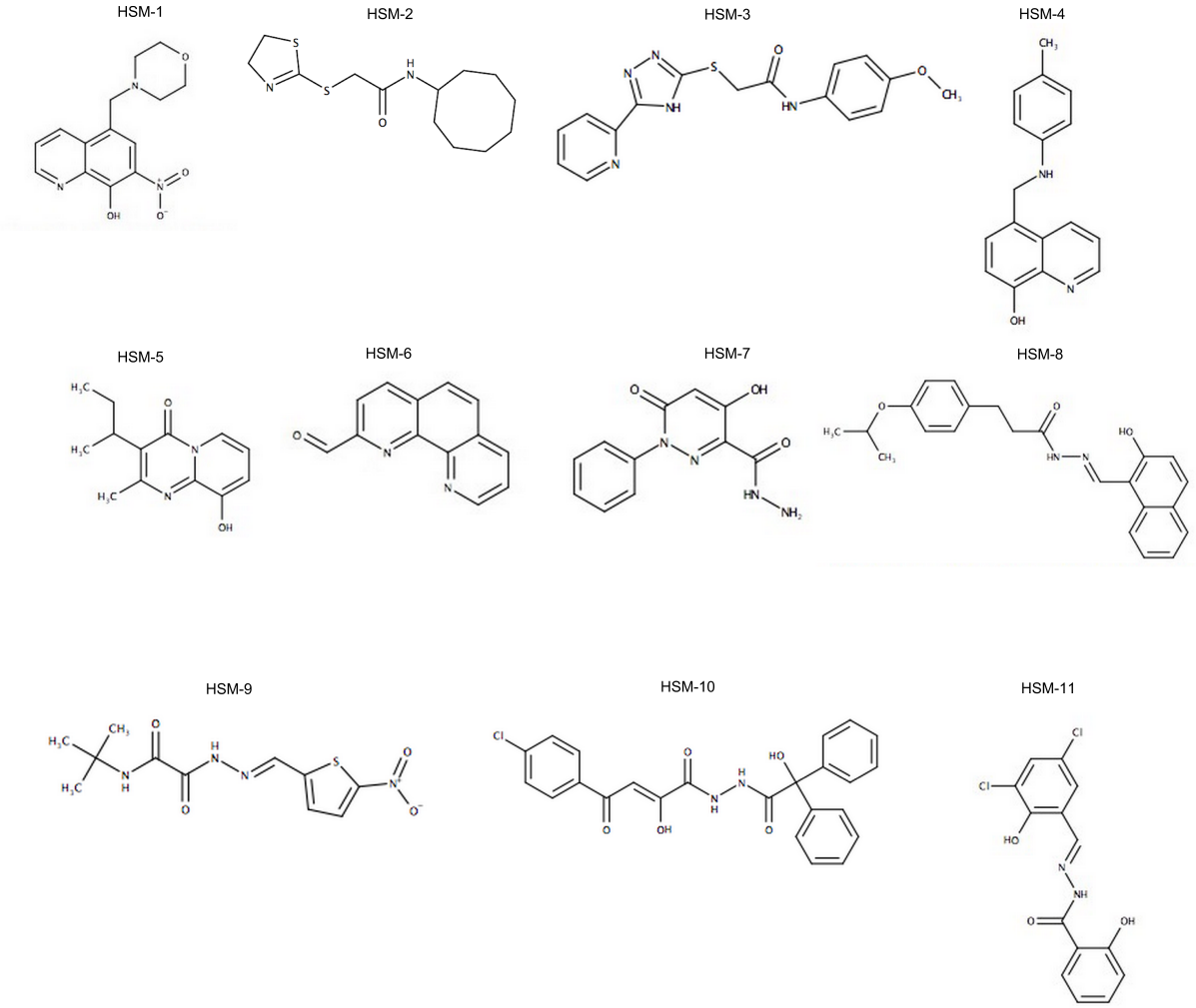


Figure 6. The chemical structures of the eleven candidate small molecules.

Table 1. Summary of cytotoxicity (CC50) and inhibition on HIV-1 replication (IC50) by the candidate small molecules.

	Small molecule	CC50 average[a] (μM)	IC50 average[b] (μM)	Selective Index[c]
HSM-1	5-(morpholin-4-ylmethyl)-7-nitroquinolin-8-ol	82.0 ± 10.9	No effect	
HSM-2	N-cyclooctyl-2-(1,3-thiazolin-2-ylthio)acetamide	59.7 ± 5.2	No effect	
HSM-3	N-(4-methoxyphenyl)-2-(5-(2-pyridyl)(4H-1,2,4-triazol-3-ylthio))acetamide	32.4 ± 5.4	NT[d]	
HSM-4	5-{[(4-methylphenyl)amino]methyl}quinolin-8-ol	50.1 ± 12.6	No effect	
HSM-5	3-(butan-2-yl)-9-hydroxy-2-methyl-4H-pyrido[1,2-a]pyrimidin-4-one	5.7 ± 0.4	NT	
HSM-6	pyridino[3,2-h]quinoline-2-carbaldehyde	6.8 ± 0.2	NT	
HSM-7	4-hydroxy-6-oxo-1-phenyl-1,6-dihydropyridazine-3-carbohydrazide	96.8 ± 4.5	No effect	
HSM-8	N'-[(1E)-2-(2-hydroxynaphthyl)-1-azavinyl]-3-[4-(methylethoxy)phenyl]propanamide	1.4 ± 0.5	NT	
HSM-9	N'-[(1E)-2-(5-nitro(2-thienyl))-1-azavinyl]-N'-(tert-butyl)ethane-1,2-diamide	45.7 ± 12.1	1.5 ± 0.01	30.5
HSM-10	(2Z)-4-(4-chlorophenyl)-2-hydroxy-N-(2-'(2Z)-4-(4-chlorophenyl)-2-hydroxy-N-(2-hydroxy-2,2-diphenylacetylamino)-4-oxobut-2-enamide-2,2-diphenylacetylamino)-4-oxobut-2-enamide	56.2 ± 4.0	9.8 ± 1.40	5.7
HSM-11	N'-[(1E)-2-(3,5-dichloro-2-hydroxyphenyl)-1-azavinyl](2-hydroxyphenyl)carboxamide	0.9 ± 0.1	NT	

a. The CC50 of each candidate small molecule was determined by WST-1 assay. CD4+ cell line CEM cells (1 x 10^3 cells/well) were cultured in RPMI 1640 and treated with serially diluted candidate small molecules (0 to 100 μM) in 96-well plates for 4 days.

b. The IC50 of each candidate small molecule was determined by HIV-I infection assay. CEM cells (2.5 x 10^4 cells/well) were infected with HIV-1 NL4-3 and small molecules (0 to 20 μM). Amount of virus in supernatants was measured by p24 antigen ELISA at 4 days after infection.

c. The selective Index (SI) = CC50/IC50

d. NT: not tested

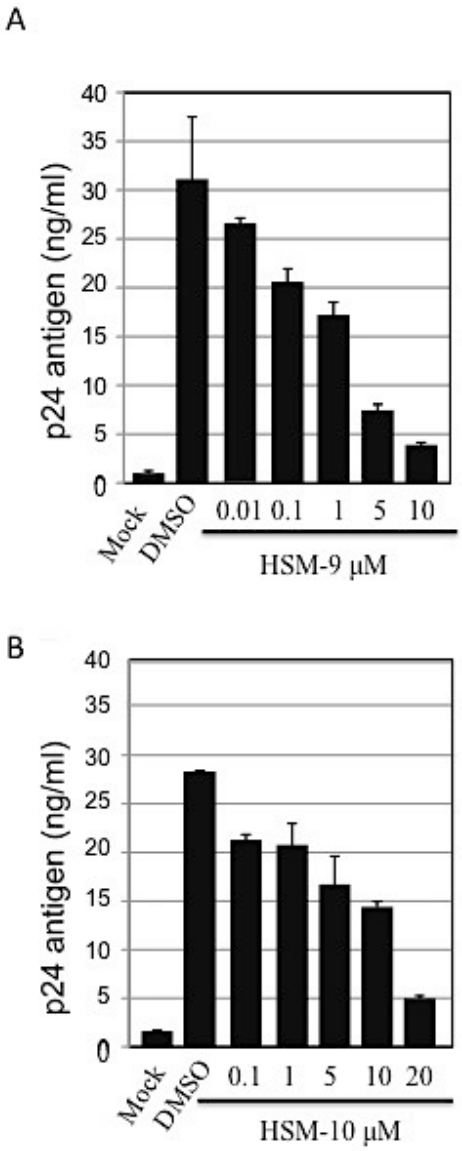


Figure 7. The effect of the hit small molecules, HSM-9 and HSM-10 on HIV-1 replication. CEM cells (2.5 x 10^4 cells/well) were infected with HIV-1 NL43-2 and treated with serially diluted small molecules (0 to 20 μM) or DMSO. The amount of virus in the supernatants was measured by p24 antigen ELISA at 4 days after infection. Each column and error bar represent the mean ± SD of results from three samples.

Table 2. Summary of results from screening 9,600 small molecules.

Screening Method	Target	Hit small molecules
-	-	9600
ELISA-binding assay I	Gag-TSG101 interaction	81
ELISA-binding assay II	Gag-TSG101 interaction	11
WST-1 Assay	Cell viability	6
HIV-1 Infection Assay	HIV-1 replication	2

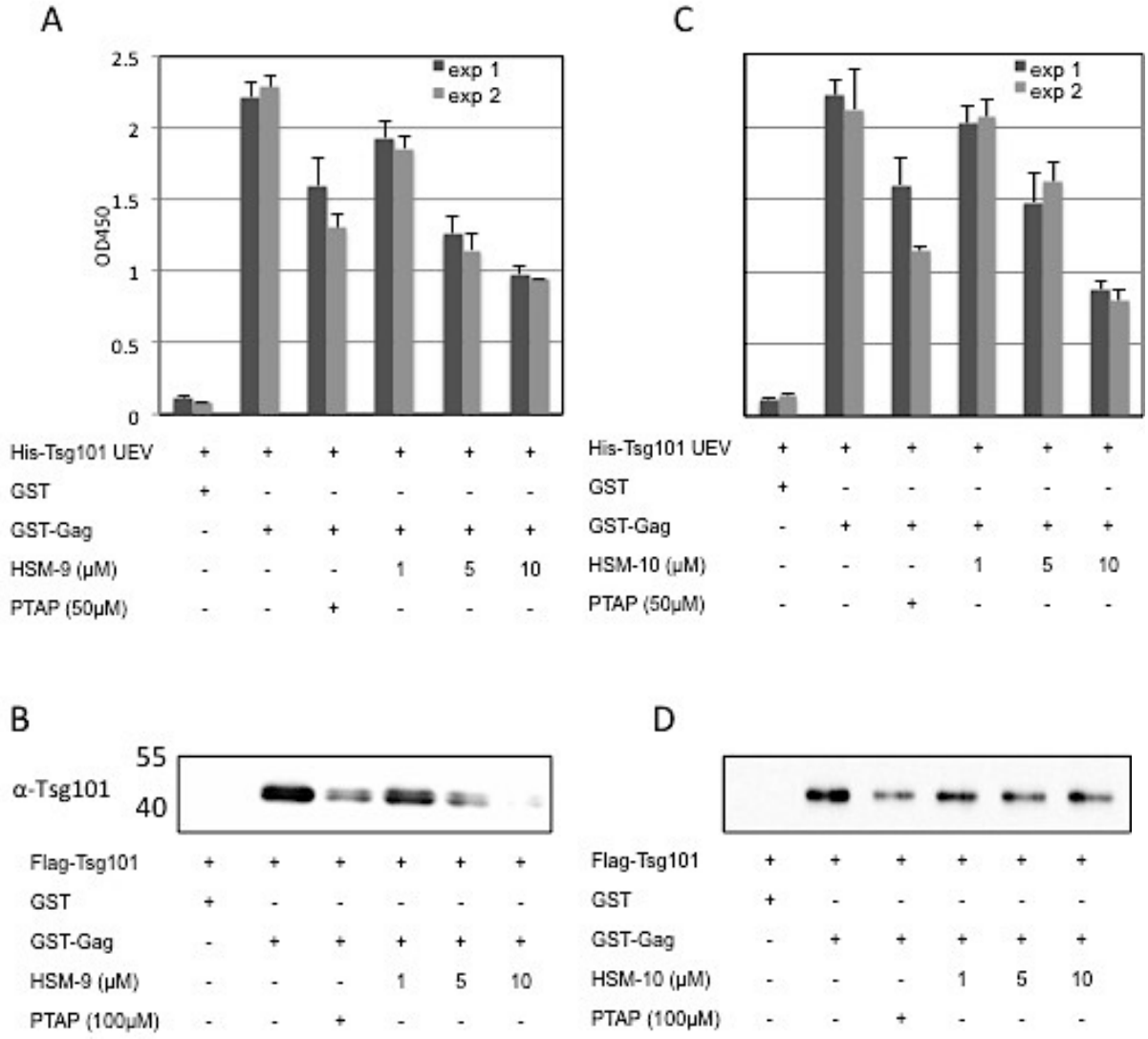


Figure 8. Inhibition of Gag-TSG101 binding by the hit small molecules, HSM-9 and HSM-10. A, C) ELISA-based binding assay. Purified GST-Gag of Gag are incubated with His-TSG101 UEV in the absence (-), or presence of 1, 5, and 10 μM of hit small molecules or 50 μM of PTAP. Each column and error bar represent the mean ± SD of results from three samples in two independent experiments. B, D) GST pull down. Glutathione sepharose beads coupled with GST-Gag or GST were incubated with Flag-TSG101 in the absence (-) or presence of 1, 5, 10 μM small molecule or 100 μM of PTAP peptide. The complexes recovered were analyzed by Western blotting with anti-TSG101 mAb. Position of molecular mass markers (kDa) are indicated.

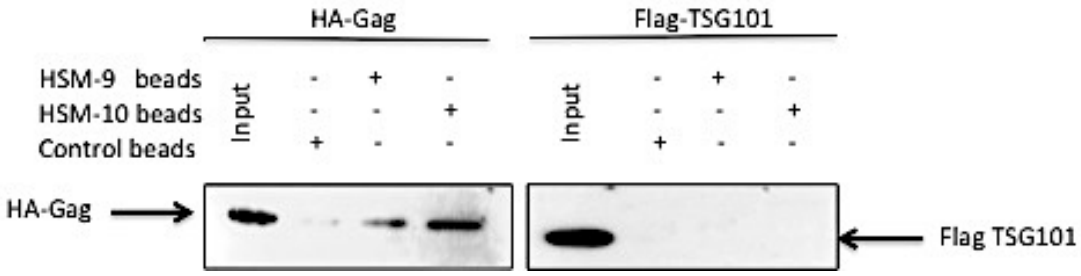
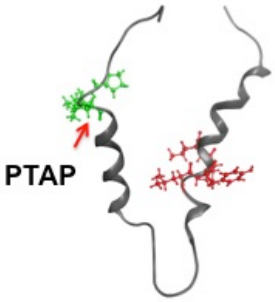


Figure 9. Photo-cross-linked small molecule affinity beads assay. Purified HA-Gag and FLAG-TSG101 proteins were mixed with photo-cross-linked HSM-9, HSM-10 and control beads, and then beads were incubated at 4°C for 24 h. The proteins bound to the small molecule and 1/2 of the input purified proteins were detected by Western blot analysis with anti-Gag mAb or anti-TSG101 mAb.

A



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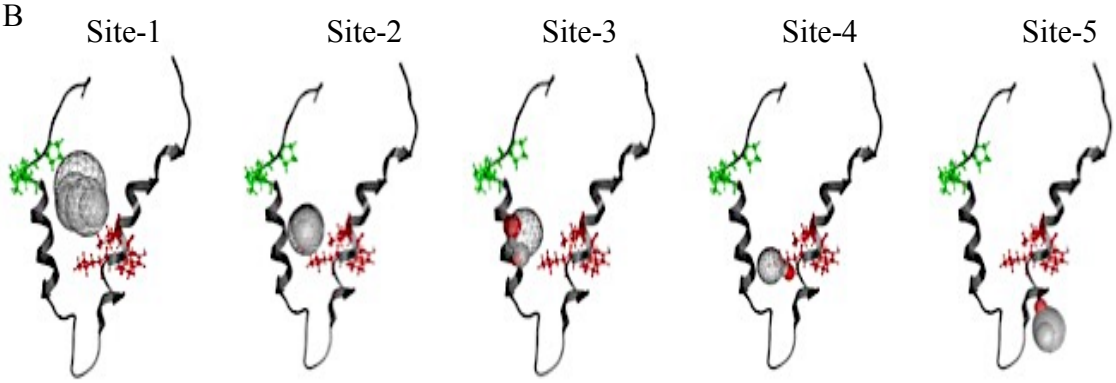

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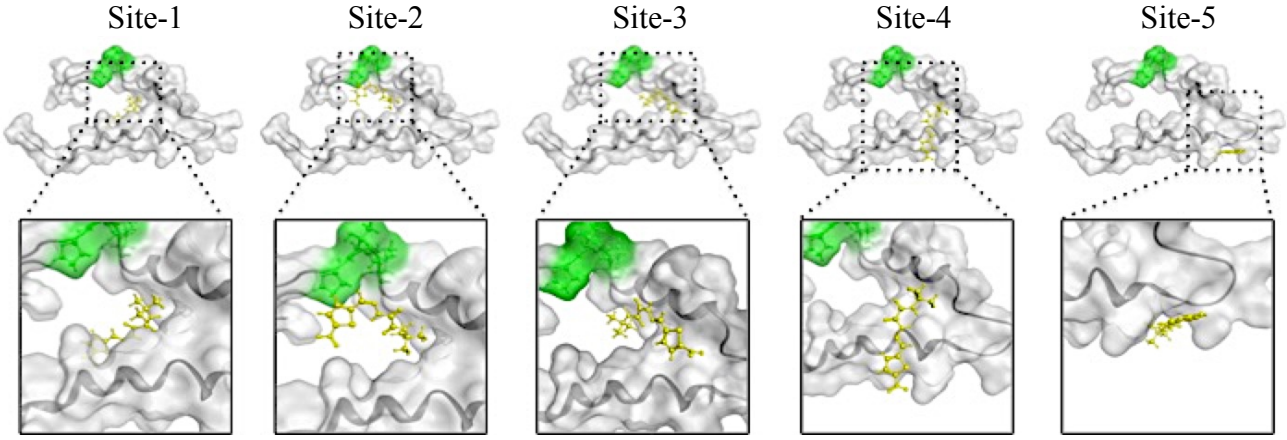
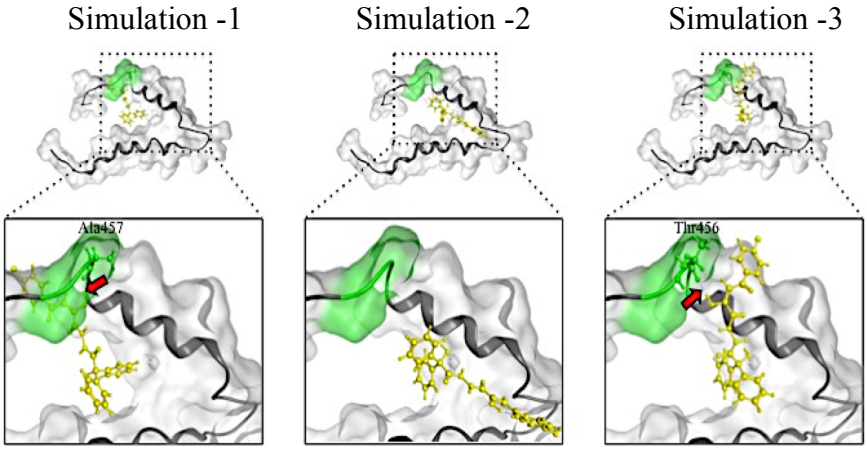


HSM-9: Simulation-1

D



HSM-10: Site-3

Figure 10. *In silico* analysis of docking sites of interaction between HIV-1 Gag p6 and h hit small molecules, HSM-9 and HSM-10. (A) The structure of HIV-1 Gag p6 protein (PDB ID: 2c55) is shown with information on PTAP motif (green), which interacted with TSG101.(B) The five candidate binding sites (sphere) found around the Gag p6 are highlighted. (C) Docking simulation complex models of HIV-1 Gag p6 protein and HSM-9. Each model with highest docking score (Site-1; -22.72 kcal/mol. Site-2; -16.19 kcal/mol. Site-3; -20.87 kcal/mol. Site-4; -23.28 kcal/mol. Site-5; -23.13 kcal/mol.) are shown. Upper panels; HIV-p6 protein (gray) with PTAP region (green) and HSM-9 (yellow). Lower panels; Close-up view around the HIV-p6 protein (gray) and HSM-9 (yellow). (D) Docking simulation complex models of HIV-1 Gag p6 protein and HSM-10. Three models with high docking scores (Simulation 1; -30.10 kcal/mol, Simulation 2; -25.94 kcal/mol. Simulation 3; -24.95 kcal/mol) are shown. Upper panels; Gag p6 protein (gray) with PTAP (green) and HSM-10 (yellow). Lower panels; Close-up view around the PTAP (green) and HSM-10 (yellow). A red arrow indicates the hydrogen bond found between the amino acid residue of main chain in PTAP (Simulation 1; alanine, Simulation 3; threonine) and HSM-10.

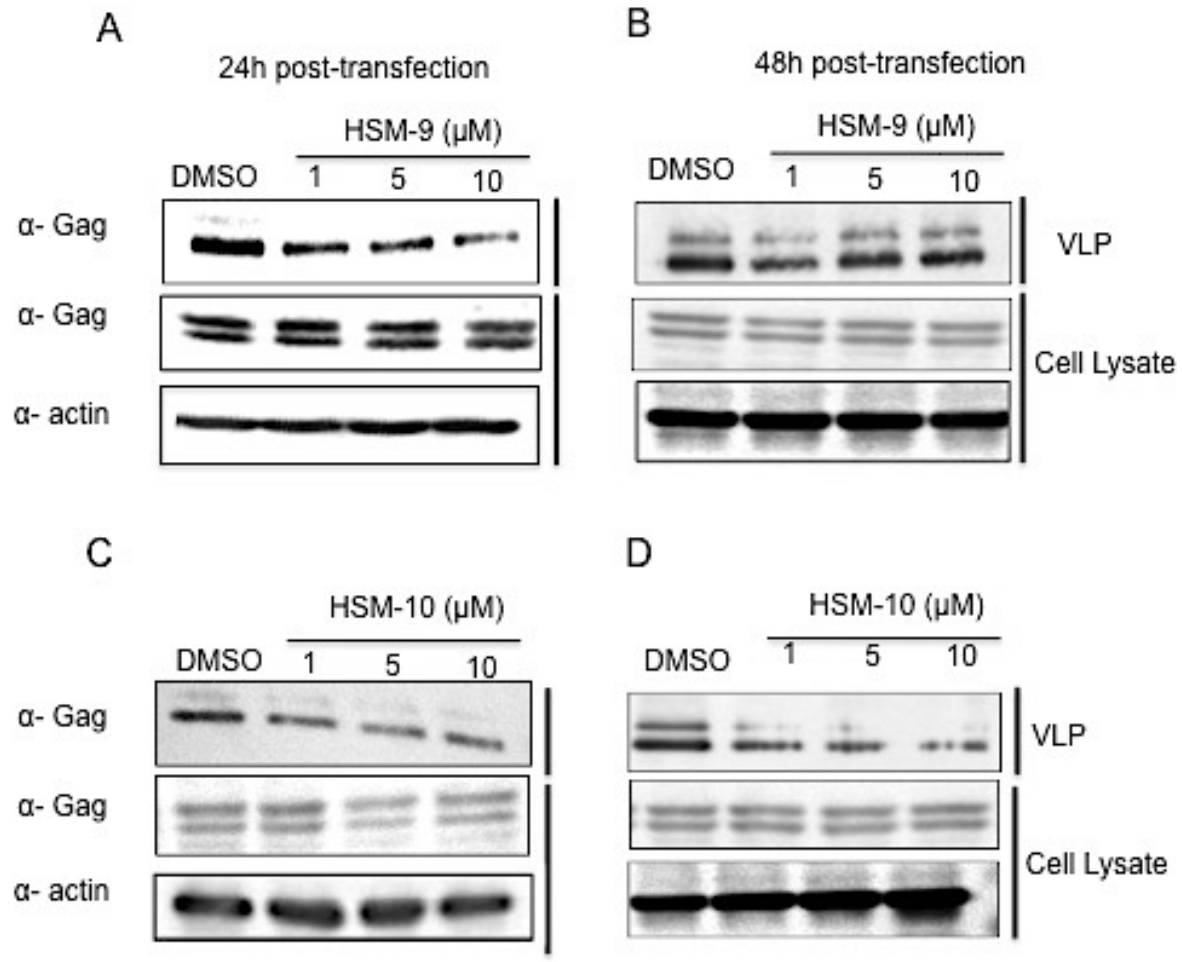


Figure 11. Gag VLP release assay. 293T cells were transfected with pCAGGS Gag and treated with varying concentrations of candidate small molecules, HSM-9 (A and B) and HSM-10 (C and D). After 24 h (A and C) and 48 h (B and D) incubation, VLPs in the cultured medium were collected by 20% sucrose cushion and whole cell lysates were also prepared. All samples were subjected to Western blot analysis with anti-Gag and anti-β-actin mAbs.

Table 3. The docking scores for evaluating interactions between HIV-1 p6 protein and small molecules.

Small molecules	candidate	ASE Dock Simulation (with Site 1-5)									
		Site-1		Site-2		Site-3		Site-4		Site-5	
	top 3	U-dock	H-bond	U-dock	H-bond	U-dock	H-bond	U-dock	H-bond	U-dock	H-bond
HSM-1	1	-17.53	-	-24.24	-	-17.33	-	-19.30	-	-20.45	-
	2	-16.84	-	-21.65	-	-14.44	-	-14.44	-	-16.61	-
	3	-16.00	-	-18.76	-	-14.31	-	-14.37	-	-16.00	-
HSM-2	1	-22.07	-	-21.19	ALIX (1)	-19.57	-	-17.57	-	-19.50	-
	2	-22.00	-	-20.48	-	-18.88	-	-17.49	-	-19.32	-
	3	-21.52	-	-19.63	-	-17.12	-	-17.27	-	-19.28	-
HSM-4	1	-19.07	-	-20.11	-	-16.83	-	-16.46	-	-18.67	-
	2	-18.20	-	-18.56	-	-14.74	-	-16.11	-	-15.05	-
	3	-16.77	-	-16.71	-	-14.53	-	-14.62	-	-14.62	-
HSM-7	1	-13.70	-	-19.37	-	-16.76	-	-21.00	-	-19.15	-
	2	-12.55	-	-15.00	-	-14.69	-	-14.92	-	-17.28	-
	3	-11.38	-	-13.60	-	-13.61	-	-13.85	-	-17.25	-
HSM-9	1	-22.72	-	-16.19	-	-20.87	-	-23.28	-	-23.13	-
	2	-18.88	-	-14.88	-	-20.43	-	-19.61	-	-21.86	-
	3	-15.36	-	-14.85	-	-20.00	-	-18.38	-	-18.16	-
HSM-10	1	-26.28	-	-20.41	-	-30.10	PTAP (1)	-24.15	-	-26.50	-
	2	-21.00	-	-20.05	-	-25.94	-	-22.56	-	-25.26	-
	3	-20.73	-	-19.29	-	-24.95	PTAP (1)	-19.38	-	-25.25	-

論文の内容の要旨

応用生命工学博士

平成27年度博士課程進学

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指導教員名　小柳津　広志

論文題目 Novel human immunodeficiency virus type-1 (HIV-1) inhibitors that reduce virus production via inhibition of viral Gag-host TSG101 interaction

（ヒト免疫不全ウィルス 1 型（HIV-1) Gag タンパク質と宿主因子TSG101との相互作用を標的とする新規HIV薬の開発）

Human immunodeficiency virus type-1 (HIV-1), the causative agent of HIV-1 infection and acquired immunodeficiency syndrome (AIDS) is up to now considered to be a major global health issue. Numerous studies have already been conducted in the attempt to find a curative therapy. Yet, there is neither an effective vaccine available to prevent HIV-1 infection nor an effective cure as of to date to completely eradicate the virus from an infected person. The current therapy, HAART (highly active antiretroviral therapy), which is a combination of several antiretroviral drugs, targets viral proteins involved in viral entry and fusion as well as the key enzymes involved in each stage of HIV-1 life cycle. HAART has proven to be effective as it reduced the levels of HIV-1 in an infected person. However, the treatment has to be prolonged throughout a patient's lifetime since HAART cannot completely eradicate the virus. This poses a risk of drug toxicities and a risk of emergence of resistant viruses. Thus, there is still a pressing need to search and develop a more potent antiretroviral drugs with novel targets and mechanisms of action.

One potential strategy is by interfering viral-host protein interactions of HIV-1 life cycle. HIV-I Gag p6 protein associates with the host's endosomal sorting complex required for transport (ESCRT) pathway to facilitate assembly, budding and release of progeny virions. Specifically, The PTAP motif of Gag p6 interacts with ubiquitin E2 variant (UEV) of Tumor susceptibility gene 101 (TSG101), a subunit of host cell's ESCRT machinery for efficient viral budding and release. Several studies demonstrated the negative effects of interfering this viral-host protein interaction on viral budding and release of infectious virus as well as on viral

production, suggesting that Gag-TSG101 interaction represents an appealing target for the development of anti-HIV-1 drugs.

As a continued quest for a curative and more potent HIV-1 therapy, this study aimed to identify HIV-1 inhibitors by using viral Gag-host TSG101 interaction as a drug target as well as aimed to characterize the mechanism of inhibition of the hit small molecules.

1. Development of a new HTS system using enzyme-linked immunosorbent assay (ELISA)-based binding assay

A high throughput screening (HTS) system was developed based on ELISA-binding assay. Recombinant bacterial vector that expresses either full-length HIV-1 Gag protein or the TSG101 protein with UEV domain was constructed with N-terminal GST tag and His tag, respectively. An expression vector encoding for GST protein was used as negative control. GST fusion proteins (GST and GST Gag) and His TSG101 UEV proteins were purified from BL21 *E.coli* using Glutathione Sepharose beads and His-affinity column, respectively. ELISA-based binding assay was developed by determining the optimal conditions of the assay and the optimal concentrations of the purified proteins. In this assay, purified GST (positive control) or GST-tagged Gag protein (negative control) was immobilized on the 96-well microplate. Following the washing and blocking steps, His-TSG101 UEV protein was added and incubated with DMSO (negative control) or with the small molecules. Gag-TSG101 interaction was detected by anti-His HRP monoclonal antibody and addition of TMB substrate. The strength of binding between the two proteins was measured based on absorbance values. To assess the quality of ELISA-based screening assay, absorbance values of the positive and negative controls were compared and each assay was validated based on Z' factor, a coefficient which reflects both the assay signal dynamic range and the data variation associated with the signal measurements. Using these parameters, it was observed that for every plate assay performed, Z' factor was within the required range, that is greater than 0.5 but not higher than 1.0. Absorbance value from each small molecule were normalized as percentage of inhibition on Gag-TSG101 binding relative to the average absorbance values of positive and negative controls of each assay.

2. Screening of small molecule inhibitors of viral Gag-host TSG101 protein interaction

A core library from the University of Tokyo Drug Discovery Initiative containing 9,600 diverse small molecules was screened for Gag-TSG101 inhibitors through a high throughput screening system based on ELISA-based binding assay. On the initial screening, eighty-one small molecules inhibited GST Gag-His TSG101 UEV interaction. ELISA binding assay was repeated using the primary hits to select the true primary

hits. Based on the mean from two independent ELISA-binding assays, eleven small molecules (coded as HSM-1, HSM-2, HSM-3, HSM-4, HSM-5, HSM-6, HSM-9, HSM-10 and HSM-11) inhibited Gag-TSG101 interaction by 30% to 80%. Small molecules HSM-4, HSM-9 and HSM-10 showed the highest percentage of binding inhibition at 75.5% to 80%. Chemical structures of the eleven candidate small molecules were varied and no similarity in structure was observed.

Second screening was performed by WST-1 assay to select the candidate small molecules with low cytotoxic effect on $CD4^+$ cell line, CEM cells. Small molecules with 50% CEM cell viability greater than 40 μM were selected and used for the next screening assay. Among the eleven candidate small molecules that inhibited Gag-TSG101 interaction, six of these (HSM-1, HSM-2, HSM-4, HSM-7, HSM-9 and HSM-10) were observed to have 50% CEM cell viability (CC50) at concentrations greater than 40 μM.

Given that the Gag-TSG101 binding is indispensable in HIV-1 life cycle particularly in the viral assembly and release, interfering this binding would presumably block HIV-1 replication. To further select the candidate small molecules with antiretroviral activity, a third screening was performed through HIV-1 infection assay. $CD4^+$ cells, CEM infected with HIV-1 NL43-2 were cultured with the serially diluted candidate small molecules. Virus replication was assessed using the supernatants of cell culture by p24 ELISA at 4 days post infection. Interestingly, supernatants from HIV-I infected cells treated with HSM-9 and HSM-10 showed a decrease in p24 concentration in a dose dependent manner at 4 days post infection. Both HSM-9 and HSM-10 strongly inhibited the viral replication and reduced the p24 value to 50% (IC50) at 1.5 μM and 9.8 μM, respectively. This strongly suggests that inhibition of Gag-TSG101 interaction could apparently block HIV-1 replication. Comparing the selective index (SI) between these small molecules, the SI of HSM-9 is more than 5 fold higher than that of HSM-10. Taken together, successive screening of 9,600 small molecules revealed that HSM-9 and HSM-10 small molecules, which strongly blocked Gag-TSG101 interaction, also potently inhibited HIV-I replication.

2. HSM-9 and HSM-10 inhibit Gag-TSG101 interaction *in vitro*

Both HSM-9 and HSM-10 small molecules showed the highest percentage of inhibition in Gag-TSG101 binding in ELISA-based high throughput screening assay. A similar ELISA-based binding assay was performed in the presence of increasing concentrations of each small molecule (1, 5, and 10 μM). Both HSM-9 and HSM-10 were able to inhibit the interaction between GST-Gag and His-TSG101 UEV in a dose dependent manner with EC50 6.2 ± 0.88 μM and 7.8 ± 0.28 μM, respectively. This inhibitory activity was confirmed by GST pull

down assay using GST-Gag and full length FLAG-tagged TSG101 proteins. GST or GST-Gag bound to glutathione sepharose beads was incubated with Flag-TSG101 with or without HSM-9 and HSM-10 small molecules at 1, 5, and 10 μM. Western blotting analyses showed that the binding of FLAG-TSG101 to GST-Gag decreased in a dose-dependent manner in the presence of HSM-9 or HSM-10. However, Gag-TSG101 binding was more highly inhibited when HSM-9 was added at 10 μM (>80%) while when HSM-10 was added at the same concentration, the Gag-TSG101 binding was only inhibited for less than 40%. Nonetheless, this result further confirms the dose-dependent inhibitory activity of both HSM-9 and HSM-10 on Gag-TSG101 binding.

3. HSM-9 and HSM-10 target HIV-1 Gag, but not TSG101

It is crucial to identify the specific protein partner of the hit small molecules to better understand its mechanism of actions. Small molecules were photo-cross-linked to sepharose beads and a pull-down assay was performed to determine which of the two interacting Gag-TSG101 proteins do HSM-9 and HSM-10 bind to exert its inhibitory effect. HSM-9/HSM-10 photo-cross-linked beads were incubated with either HA-tagged Gag or FLAG-tagged TSG101 and the proteins bound to the small molecule-linked beads were determined by Western blotting using anti-Gag mAb and anti-TSG101 mAb, respectively. Results from this assay show that HA-Gag proteins co-precipitated in the sample containing HSM-9 and HSM-10 affinity linked beads, indicating that HSM-9 and HSM-10 small molecules specifically bind to Gag protein and not to TSG101.

4. HSM-9 and HSM-10 have different docking sites in HIV-1 p6

To better understand the Gag-dependent inhibitory activity of HSM-9 and HSM-10, binding sites of each small molecule in HIV-1 p6 were determined by *in silico* docking simulation analysis. Five possible docking sites in HIV-1 p6 were identified and were used in the docking simulation analysis. Analysis reveals that the U-dock score of HSM-10 at site-3 is higher than the other molecule simulations (U-dock: -30.10 kcal/mol). The simulation also showed that a hydrogen bond was formed between the HSM-10 hydroxyl group and the alanine of main chain in PTAP sequence. Aside from the alanine in PTAP, docking simulation complex models of HIV-1 p6 protein and HSM-10 also identified a hydrogen bond between the threonine residue of PTAP and HSM-10. In contrast, docking simulation complex models of HIV-1 p6 protein and HSM-9 revealed the binding of HSM-9 to the five possible binding sites in HIV-1 p6 but U-dock scores from HSM-9 simulations were lower than HSM-10 simulations and no specific amino acid residue of HIV-1 p6 was able to form a hydrogen bond with

HSM-9. Collectively, these results suggest that HSM-10 showed a specific binding with the PTAP motif of HIV-1 p6 at alanine and threonine residues, while HSM-9 has at least five non-specific binding sites in HIV-1 p6, which indicate that HSM-10 interacts more strongly with HIV-1 p6 than HSM-9.

5. HSM-9 and HSM-10 inhibit Gag VLP release

Given that the interaction between HIV-1 Gag and TSG101 plays a major role in HIV-1 assembly and release, the effect of small molecules on Gag Virus-like Particle (VLP) release was investigated. Following 24 – 48 h transfection, supernatants and cell lysates from HEK 293T cells transfected with pCAGGS Gag and incubated with each candidate small molecule (1, 5, and 10 μM), were collected and analyzed by Western blotting. Interestingly, both HSM-9 and HSM-10 efficiently inhibited VLP release in a dose dependent manner. A significant decrease of Gag VLP expression was distinct in the presence of HSM-9 (1, 5, and 10 μM) at 24 h posttransfection but not at 48 h posttransfection. In contrast, a more distinct reduction in VLP production was observed at 48 h posttransfection in the presence of HSM-10 but not at 24 h transfection, which showed a slight decrease in VLP production. These results indicate that HSM-9- and HSM-10-mediated inhibition on Gag-TSG101 interaction led to interference of VLP/virus release, which presumably account for the decrease in HIV-1 replication.

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