論文の内容の要旨

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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 $\pm$ 0.88 μM and 7.8 $\pm$ 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.