Identification of Novel Inhibitors of Human Immunodeficiency Virus Type 1 Replication by In Silico Screening Targeting Cyclin T1/Tat Interaction

Takayuki Hamasaki, Mika Okamoto, Masanori Baba

Division of Antiviral Chemotherapy Center for Chronic Viral Disease, Graduate School of Medical and Dental Sciences, Kagoshima University, 8-35-1, Sakuragaoka, Kagoshima 890-8544, Japan

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Address correspondence to Dr. Masanori Baba, Division of Antiviral Chemotherapy, Center for Chronic Viral Disease, Graduate School of Medical and Dental Sciences, Kagoshima University, 8-35-1, Sakuragaoka, Kagoshima 890-8544, Japan.

Phone: 81-99-275-5930, Fax: 81-99-275-5932,

E-mail: m-baba@m2.kufm.kagoshima-u.ac.jp
Human immunodeficiency virus type 1 (HIV-1) transcription is essential for viral replication and the only step for viral genome amplification. Cyclin T1 (CycT1) interacts with HIV-1 Tat and transactivation-responsive (TAR) RNA, leading to the activation of viral transcription through the hyperphosphorylation of RNA polymerase II (RNAPII). Thus, the CycT1/Tat/TAR RNA interaction represents a novel target for inhibition of HIV-1 replication. In this study, we conducted in silico screening of compounds targeting the CycT1/Tat/TAR RNA complex and found that two structurally related compounds (C1 and C2) had high docking scores for a model of the complex. These compounds proved inhibitory to HIV-1 replication in tumor necrosis factor α-stimulated chronically infected cells. In addition, C3, a derivative of C1 and C2, was found to be a more potent inhibitor of HIV-1 replication in chronically infected cells. C3 also inhibited HIV-1 replication in acutely infected cells. The compound could suppress the Tat-mediated HIV-1 long terminal repeat-driven gene expression and phosphorylation of RNAPII through the inhibition of Tat binding to CycT1. Furthermore, the docking pose of C3 was defined by the analyses for its in silico docking energy and in vitro antiviral activity, which indicates that C3 interacts with Tat-binding amino acids of CycT1. Thus, a series of compounds described herein is novel inhibitors of HIV-1 transcription through the inhibition of CycT1/Tat interaction.
Introduction

The current antiretroviral therapies (ART) against human immunodeficiency virus type 1 (HIV-1) have proved highly effective in reducing viral load and delaying disease progression in infected patients (30). However, even such effective therapies cannot completely eradicate the virus from their bodies, and treatment interruption generates a rebound of viral load from certain reservoir cells chronically infected with HIV-1 (26). Thus, infected patients need to be continuously treated with antiretroviral drugs for a long period of time, presumably, throughout their life, which leads to serious concerns about the emergence of drug-resistant viruses and chronic adverse effects of the drugs. Considering the fact that effective vaccines against HIV-1 are not available (21), the developments of novel antiretroviral drugs with a different mechanism of action are still mandatory.

Transcription of HIV-1 genome RNA from its proviral DNA is a crucial step in the viral life cycle, and the amplification of genetic information occurs at only this step. HIV-1 transcription is predominantly controlled at the step of RNA elongation by the virus-encoded transcriptional activator protein Tat (7, 22, 25). Tat directly binds to Cyclin T1 (CycT1), a subunit of positive transcription elongation factor b (P-TEFb), which is composed of CycT1 and cyclin-dependent kinase 9 (CDK9) (33). Tat recruits P-TEFb to transactivation-responsive (TAR) RNA located at the 5’ end of nascent HIV-1 transcripts (6, 19, 25, 27, 33). Subsequently, the CDK9 subunit of P-TEFb phosphorylates Ser2 of the heptad repeats in the C-terminal domain (CTD) of RNA polymerase II (RNAPII), which is a marker of the transcriptional transition from initiation to elongation. The phosphorylated RNAPII starts the elongation of HIV-1
transcripts. Thus, the complex formation of P-TEFb/Tat/TAR RNA is essential for the amplification of HIV-1 genome RNA, and their interfaces are considered to be target sites for novel intervention in HIV-1 transcription.

Human CycT1 is comprised of 726 amino acids and contains a cyclin box repeat domain (31–250), a coiled-coil sequence (379–530), and a PEST sequence (709–726) (24, 33). The N-terminal amino acids (1–272) of CycT1 are sufficient to bind Tat and TAR RNA and to mediate transactivation by Tat (14). A previous study of mutant CycT1 demonstrated that the Tat/TAR RNA recognition motif (TRM) of CycT1 (250–262) was essential for the CycT1/Tat/TAR RNA complex formation. In particular, N250, R259, and C261 of the TRM were crucial for Tat binding, while R251, L252, R254, I255, and W258 were required for TAR RNA binding (15). Thus, the TRM region of CycT1 is a possible target of compounds for inhibition of HIV-1 transcription. In addition, two crystallographic structures of CycT1 with viral factor(s) have recently been reported, such as the equine CycT1/equine infectious anemia virus (EIAV) Tat/TAR RNA complex [Protein Data Bank (PDB) ID: 2W2H] (2) and human CycT1/HIV-1 Tat complex (PDB ID: 3MIA) (29). The structures of these complexes provide the interactive and structural information between CycT1 and viral factor(s) in further detail. Although the structure of human P-TEFb/HIV-1 Tat reported by Tahirov et al. revealed the interface between CycT1 and Tat, the TRM could not be elucidated because of its distorted structure (29). On the other hand, Anand et al. demonstrated the structure of equine CycT1/EIAV Tat/TAR RNA including the TRM region, in which the TRM of equine CycT1 interacted with EIAV Tat but not with EIAV TAR RNA in this complex (2). Thus, although the structure of human CycT1/HIV-1 Tat/TAR RNA has not fully been clarified yet, these two structure models provide useful information on
possible targets of small-molecule compounds for intervention in HIV-1 transcription.

There are several reports on the identification of small-molecule inhibitors of HIV-1 transcription, and most of the inhibitors target CDK9 or TAR RNA (9, 10, 12, 31). We have also reported two compounds, K-37 (4) and JTK-101 (32), as potent and selective inhibitors of HIV-1 transcription. As mentioned above, the interfaces between CycT1 and viral factor(s) have recently been elucidated at a molecular level (2, 29). Therefore, it seems important to determine whether these interfaces can be used for identifying novel anti-HIV-1 agents. In this study, we conducted \textit{in silico} screening of 3,000,000 compounds targeting the TRM of human CycT1 based on the structure of equine CycT1/EIAV Tat/TAR RNA and found that some compounds selected by the screening also inhibited HIV-1 replication \textit{in vitro}. The most active compound proved to suppress the Tat-mediated HIV-1 long terminal repeat (LTR)-driven gene expression and phosphorylation of RNAPII through the inhibition of Tat binding to CycT1.
Materials and methods

Cells. OM-10.1 (8), U1 (13), CEM (32), MOLT-4 cells (20), and peripheral blood mononuclear cells (PBMCs) were used in the anti-HIV-1 assays. OM-10.1 and U1 cells are clones of HL-60 and U937 cells latently infected with HIV-1, respectively. PBMCs were obtained from healthy donors and stimulated with phytohemagglutinin (PHA) (Sigma-Aldrich, Saint Louis, MO). W-3 and KM-3 cells were used for a reporter assay. W-3 and KM-3 cells are clones of CEM cells that stably integrate an HIV-1 LTR-driven secreted alkaline phosphatase (SEAP) gene (5). The integrated HIV-1 LTR contains two intact nuclear factor (NF)-κB-binding sites in W-3 cells, whereas both of the sites are mutated in KM-3 cells.

Compound database and conformer generation. A compound database containing approximately 3,000,000 molecules was obtained from Namiki, Tokyo, Japan. All in silico studies were performed using Molecular Operating Environment (MOE) software (Chemical Computing Group, Montreal, Canada). To select drug-likeness compounds, the database was filtered with the following conditions; molecular weight: 350–600, logP: 0–6, the number of hydrogen bond donor/acceptor: < 13, and the number of rotatable bond: < 7. Partial charges were added to compounds, and at maximum 250 conformers per one compound were generated using the force field, Merck Molecular Force Field 94X (MMFF94x) (17, 18, 34).

In silico screening of compounds. Using MOE software, a model structure of human CycT1 was constructed by homology modeling based on the X-ray crystal structure of a
complex composed of equine CycT1, EIA V Tat, and EIA V TAR RNA (PDB ID: 2W2H) (2), which were available from PDB at the Research Collaboration for Structural Bioinformatics (http://www.rcsb.org/pdb/home/home.do). After adding hydrogen atoms and assigning atomic charges, the constructed human CycT1 model was subjected to energy minimization using the force field MMFF94x (17, 18, 34). Alpha Site Finder, a function of MOE, was used to search the target sites for \textit{in silico} screening, where compounds could bind, in the human CycT1 model. We selected a pocket containing the TRM of CycT1 as a target site. MOE-ASEDock 2005 (Ryoka Systems, Tokyo, Japan) (16) was used for docking drug-likeness compounds to the target site. The docking state was evaluated by the docking energy calculated by MOE software, and compounds with high docking energy scores were selected and purchased from Namiki for further evaluation \textit{in vitro}.

\textbf{Anti-HIV-1 assays.} The anti-HIV-1 activity of test compounds in chronically infected cells was based on the inhibition of HIV-1 p24 antigen production in OM-10.1 and U1 cells stimulated with tumor necrosis factor (TNF)-\(\alpha\) (Roche Diagnostic, Mannheim, Germany). Briefly, OM-10.1 and U1 cells (1 \(\times\) 10^5 cells/ml) were incubated in the presence of various concentrations of the compounds for 24 h and stimulated with 0.1 ng/ml TNF-\(\alpha\). After incubation for 3 days at 37°C, the culture supernatants were collected, and their p24 antigen levels were determined with a sandwich enzyme-linked immunosorbert assay (ELISA) kit (ZeptoMetrix, Buffalo, NY). The anti-HIV-1 activity of the compounds in acutely infected cells was based on the inhibition of p24 antigen production in CEM cells, MOLT-4 cells, and PBMCs infected with HIV-1 (IIIb strain). CEM and MOLT-4 cells were infected with HIV-1 at a multiplicity of infection (MOI)
of 0.001, while PBMCs were infected with HIV-1 at a MOI of 0.01. After viral adsorption for 2 h, the cells were washed thoroughly with culture medium to remove unabsorbed viral particles. The infected cells (1 × 10^5 cells/ml) were cultured in the presence of various concentrations of the compounds. After incubation for 3 days at 37ºC, the cells were subcultured at a ratio of 1:5 with fresh medium containing appropriate concentrations of the compounds and further incubated for 3 days. The cytotoxicity of the compounds were evaluated in parallel with their antiviral activity, which was based on the reduction of the viability of TNF-α-untreated OM-10.1 and U1 cells or mock-infected CEM cells, MOLT-4 cells, and PBMCs by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method, as previously described (23). The EC_{50} and CC_{50} values were determined by the median-effect method (11).

**Reporter assays.** W-3 and KM-3 cells (1 × 10^5 cells/ml) were either treated with 10 ng/ml TNF-α (Roche Diagnostic) or transfected with 0.2 µg of an HIV-1 Tat-expression plasmid containing the second exon under the control of the simian virus 40 promoter (modification of pSV2tat72) by Gene Pulser® II (300 V; 1,000 µF, Bio-Rad Laboratories, Hercules, CA) (5). The cells were cultured in the presence of various concentrations of the compounds. After incubation for 24 h at 37ºC, the culture supernatants were collected, incubated for 30 min at 65ºC to inactivate the alkaline phosphatase activity of fetal bovine serum in culture medium, and examined for their SEAP levels using GreatEscape SEAP detection kit (Clontech, Palo Alto, CA). The chemiluminescent intensity was measured by TriStar Multimode Microplate Reader LB 941 (Berthold Technologies, Bad Wildbad, Germany). At the same time, the viable cell number was determined by a dye method using a water soluble tetrazolium, Tetracolor One®.
Western blot analysis. W-3 cells (1 × 10^5 cells/ml) were transfected with 0.2 µg of the Tat-expression plasmid and cultured in the presence of various concentrations of the compounds. After incubation for 2 days at 37°C, the cells were washed and lysed with an assay buffer for immunoprecipitation (Nacalai Tesque, Kyoto, Japan). The cell lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 10% gel, and the separated proteins were electrophoretically transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore, Billerica, MA) using a semidy transfer apparatus (Bio-Rad Laboratories). The membrane was incubated with anti-phosphorylated RNAPII (Ser2) (Novus Biologicals Inc., Littleton, CO), anti-RNAPII (Novus Biologicals), and anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. After washing three times with phosphate-buffered saline containing Tween 20 (0.1 %) (PBS-T), the membrane was further incubated with a horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (Cell Signaling Technology, Danvers, MA) or HRP-conjugated anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). The membrane was washed three times with PBS-T and analyzed for chemiluminescence by Chmi-Lumi One (Nacalai Tesque).

Immunoprecipitation. Dynabeads Protein G (Invitrogen Dynal, Oslo, Norway) immobilized with an anti-CycT1 antibody (Santa Cruz Biotechnology) or goat IgG (Jackson ImmunoResearch, West Grove, PA) was incubated with CEM cell lysate for 1 h at 4°C. Tat (Immuno Diagnostics, Woburn, MA) was reconstituted in binding buffer

(Seikagaku Corporation, Tokyo, Japan).
[50 mM Tris-HCl (pH 7.5) and 0.5 % NP-40] containing 120 mM NaCl, 400 µM ZnSO₄, and 1 mM DTT for 1 h at room temperature. The beads were washed with binding buffer containing 120 mM NaCl and incubated in the presence of various concentrations of C3 and the reconstituted Tat in binding buffer containing 120 mM NaCl and 10 % BSA. After incubation for 2 h at 4°C, the beads were washed three times with binding buffer containing 500 mM NaCl at room temperature. The beads were boiled in SDS sample buffer, and the supernatant was subjected to SDS-PAGE with 15% gel. Western blot analysis was performed with anti-CycT1 (Santa Cruz Biotechnology), anti-CDK9 (Santa Cruz Biotechnology), and anti-Tat (ImmunoDiagnostics) antibodies.
Results

In *silico* screening of compounds. To identify small-molecule inhibitors of HIV-1 transcription, we decided to perform *in silico* screening of compounds targeting the TRM of CycT1, which interacts with Tat and TAR RNA. To this end, we constructed a homology model of human CycT1 based on the structure of equine CycT1/EIAV Tat/TAR RNA complex. When the target sites for *in silico* screening were searched in the constructed human CycT1 model using MOE software, one site that included the TRM and might interact with small molecules was found. This site was composed of 21 amino acids of CycT1, Q162, L163, R165, A166, S167, D169, L170, Q172, F176, V199, C200, L203, A204, K206, L234, L237, T238, N250, K253, W258, and R259 (Fig. 1). Among these, N250 and R259 interact with Tat, and W258 binds to TAR RNA (15). Q172 and F176 do not belong to the TRM, but these amino acids interact with Tat in the crystallographic structure of human CycT1/Tat complex (29). We conducted *in silico* screening of compounds for this site, and the obtained data were sorted by docking energy scores calculated with MOE software. Then, 124 compounds with high docking energy scores were chosen and examined for their inhibitory effect on HIV-1 replication in TNF-α-stimulated OM-10.1 cells. Among them, two structurally related compounds (C1 and C2) (Fig. 2) proved inhibitory to HIV-1 replication in a dose-dependent manner (Fig. 3A and 3B). The 50% effective concentrations (EC50s) of C1 and C2 were 4.2 and 4.8 µM, respectively. Both compounds did not show any cytotoxicity at concentrations up to 25 µM.

Anti-HIV-1 activity *in vitro*. To find more potent compounds, derivatives of C1 and C2
were searched using PubChem (http://pubchem.ncbi.nlm.nih.gov/), and 8 compounds (C3–C10) were identified (Fig. 2). While C4–C10 did not inhibit HIV-1 replication in TNF-α-stimulated OM-10.1 cells (data not shown), C3 was found to be a more potent inhibitor of HIV-1 replication than C1 and C2 (Fig. 3C). Its EC50 was 617 ± 11 nM (Table 1). C3 also inhibited HIV-1 replication in TNF-α-stimulated U1 cells with an EC50 of 168 ± 97 nM (Fig. 3D). C3 reduced the viability of OM-10.1 and U1 cells by 53 and 56% of that of the control cells at the concentration of 10,000 nM, respectively (Fig. 3C and 3D). The selective index (SI), based on the ratio of 50% cytotoxic concentration (CC50) to EC50, was more than 16 and 60 in OM-10.1 and U1 cells, respectively (Table 1).

In the next experiment, the anti-HIV-1 activity of C3 was examined in CEM cells, MOLT-4 cells, and PBMCs, all of which were acutely infected with HIV-1. As shown in Fig. 4, C3 selectively inhibited HIV-1 replication, irrespective of the cells used for assays. Its EC50s were 19.9 ± 3.4 nM for CEM cells and 17.9 ± 6.6 nM for MOLT-4 cells, while the CC50s were more than 1,000 nM in both cell lines. Thus, the SIs were more than 50 and 55 in CEM and MOLT-4 cells, respectively (Table 1). When the cytotoxicity of C3 was determined after 3 weeks of cultivation, its CC50 was unchanged (data not shown). Furthermore, C3 inhibited HIV-1 replication in PBMCs with an EC50 of 9.6 nM. Since its CC50 for PBMCs was 6,800 nM, the SI resulted in 704. C3 was also active against HIV-2 replication. Its EC50s against HIV-2 (ROD strain) were 136.5 ± 7.9 and 110.5 ± 2.7 nM in CEM and MOLT-4 cells, respectively (data not shown).

Inhibitory effect on Tat-mediated transcription. To gain insight into the mechanism of action, C3 was examined for its inhibitory effect on NF-κB- or Tat-mediated HIV-1
LTR-driven gene expression in reporter cells. W-3 cells are reactive to stimulation with both TNF-α and Tat, since the cells carry a reporter (SEAP) gene with two intact NF-κB-binding sites in the HIV-1 LTR as a promoter. In contrast, KM-3 cells having two mutated NF-κB-binding sites are not reactive to TNF-α but fully reactive to Tat. In the absence of C3, TNF-α induced approximately 4-fold increase of SEAP activity in the culture supernatants of W-3 cells, but no such increase of SEAP activity was observed in KM-3 cells (data not shown). On the other hand, transfection with the Tat-expression plasmid induced 4-fold increase of SEAP activity in both W-3 and KM-3 cells (data not shown). Although C3 did not affect the SEAP activity of the culture supernatants in TNF-α-treated W-3 cells (Fig. 5A), dose-dependent decrease of the SEAP activity was observed in W-3 and KM-3 cells transfected with the Tat-expression plasmid (Fig. 5B and 5D). The EC_{50}s of C3 for SEAP were 183 ± 50 nM for W-3 cells and 107 ± 11 for KM-3 cells. Its CC_{50}s were more than 2,000 nM in both cell lines (Table 2). These results indicate that C3 specifically inhibited the Tat-mediated HIV-1 LTR-driven transcription.

Inhibitory effect on Tat-induced phosphorylation of RNAPII. HIV-1 Tat recruits P-TEFb (CDK9/CycT1) to TAR RNA on nascent HIV-1 transcripts, and the CDK9 subunit of P-TEFb phosphorylates Ser2 on the heptad repeats in the CTD of RNAPII to induce HIV-1 transcription (19, 25, 27). To investigate the inhibitory effect of C3 on the phosphorylation of Ser2 during transcription, Western blot analysis was conducted for the lysate from W-3 cells which had been transfected with the Tat-expression plasmid and cultured in the presence of C3. While the phosphorylation of Ser2 was detected in the absence of Tat, the transfection with the Tat-expression plasmid augmented the...
phosphorylation by 1.7-fold (Fig. 6). When the cells were exposed to C3, the phosphorylation of Ser2 was suppressed in a dose-dependent fashion without affecting the total RNAPII protein level (Fig. 6). These results suggest that C3 suppresses the Tat-induced phosphorylation of RNAPII and inhibits HIV-1 transcription before transition from the initiation step to the elongation step.

**Inhibitory Effect on CycT1-Tat interaction.** When the binding assay between Tat and CycT1 was conducted in the absence of C3, Tat could bind to CycT1 purified from CEM cells with anti-CycT1 antibody-beads. Although Tat nonspecifically bound to goat IgG-beads in this assay, the binding level was low. In the presence of C3, the binding of Tat to CycT1 was inhibited by C3 in a dose-dependent fashion, whereas the levels of CDK9 and CycT1 were not affected by the compound (Fig. 7). These results suggest that C3 specifically targets the interaction between Tat and CycT1.

**Docking pose to CycT1.** To define the docking pose of C3, we recalculated the docking energy and found two stable docking poses, docking pose 1 (Fig. 8A) and docking pose 2 (Fig. 8B). The docking pose 1 demonstrated that methoxyquinolin, pyridine, and benzothiadiazole moieties of C3 were located on the target sites 1, 2, and 3 in CycT1, respectively (Fig. 8A), while they were located on the sites 2, 1, and 3, respectively, in the docking pose 2 (Fig 8B). When the docking energy of C1–C3 was evaluated in the docking pose 1, the docking energy of C3 was significantly stronger than those of C1 and C2 (Fig. 8C). However, no significant difference of docking energy was observed between C1 and C2. Thus, the result on the docking pose 1 was consistent with that of their anti-HIV-1 activity (C3 > C1 = C2). In the docking pose 2, the docking energy
was found to differ significantly between C1 and C2 (Fig. 8D), which was inconsistent with their anti-HIV-1 activity. Thus, the docking pose 1 is more appropriate to explain the interaction between C3 and CycT1. In the docking pose 1, C3 had direct interaction with Val199, Leu203, Phe241, Lys253 and Arg259 and was located near Gln172 and Phe176 of CycT1.
Discussion

In this study, we describe a novel class of anti-HIV-1 compounds by \textit{in silico} screening targeting the TRM of CycT1. Although the transcription process of HIV-1 has not fully been exploited for therapeutic intervention yet, this process plays a crucial role in viral replication. In spite of various attempts to develop HIV-1 transcription inhibitors (3, 28), none of the inhibitors have successfully been approved for clinical use because of their potential toxicity. Since the transcription process of HIV-1 involves several host cellular factors, these compounds most likely inhibited these host factors. To circumvent such a problem, our strategy for identifying transcription inhibitors was to introduce \textit{in silico} screening of compounds targeting the TRM. Although CycT1 is a host factor essential for cellular functions, the TRM is considered to be a unique interface for Tat- and TAR RNA-binding.

As the results of \textit{in silico} screening followed by \textit{in vitro} anti-HIV-1 assay, we found two structurally related compounds (\textbf{C1} and \textbf{C2}) as selective inhibitors of HIV-1 replication in TNF-\(\alpha\)-stimulated OM-10.1 cell (Fig. 3A and 3B). We also identified \textbf{C3} as a more potent inhibitor than \textbf{C1} and \textbf{C2} by further screening. \textbf{C3} inhibited HIV-1 replication not only in chronically infected cells (OM-10.1 and U1) but also in acutely infected cells (CEM, MOLT-4, and PBMCs) (Table 1). Since Tat strongly induces HIV-1 transcription through the phosphorylation of RNAPII after recruiting the CDK9/CycT1 complex to HIV-1 LTR (6, 19, 25, 27, 33), we examined the inhibitory effect of \textbf{C3} on this process. Using a reporter expression cell system regulated under the control of HIV-1 LTR, \textbf{C3} was found to inhibit the Tat-induced reporter (SEAP) expression (Fig. 5). In addition, \textbf{C3} also inhibited the phosphorylation of Ser2 in the CTD of RNAPII.
(Fig. 6) and the binding of Tat to CycT1 (Fig. 7) in vitro. Thus, the inhibitory effect of
C3 on HIV-1 replication is probably due to the impairment of CycT1/CDK9/Tat/TAR RNA complex formation.

Although the three-dimensional structure of the human CycT1/HIV-1 Tat/TAR RNA complex has not fully been clarified, two structures, such as equine CycT1/EIAV Tat/TAR RNA (2) and human CycT1/HIV-1 Tat (29), are currently available for structure-based drug design. Since the TRM of CycT1 interacts with both Tat and TAR RNA, this region is important for the complex formation and is an attractive target for inhibition of HIV-1 replication. Since the structure of human CycT1/HIV-1 Tat complex lacks the information of the TRM, we used the equine CycT1/EIAV Tat/TAR RNA complex as a model structure of human CycT1 for *in silico* screening. The TRM of equine CycT1 directly interacts with EIAV Tat, and the amino acids involved in this interaction are highly conserved in human CycT1 and HIV-1 Tat. However, different from HIV-1 TAR RNA, EIAV TAR RNA is located far from the TRM of equine CycT1, according to the crystallographic structure of equine CycT1/EIAV Tat/TAR RNA complex. It is possible that conformational change of the TRM of equine CycT1 is induced solely by Tat binding but not by TAR RNA binding. Therefore, in spite of the possible lack of interaction between the TRM of equine CycT1 and EIAV TAR RNA, we could identify C3 as a novel inhibitor of HIV-1 transcription by *in silico* screening. These results suggest that the TRM model based on the structure of equine CycT1/EIAV Tat/TAR RNA complex adequately mimics the actual structure of the TRM of human CycT1 with viral factor(s).

In previous study, we conducted *in silico* screening of compounds targeting a site containing the TRM of human CycT1 fused with EIAV Tat (PDB ID: 2PK2) (1). Using
this system, we identified three compounds that inhibited HIV-1 replication in acutely infected cells at sub-micromolar concentrations (unpublished data). However, these compounds did not show any anti-HIV-1 activity in chronically infected cells, indicating that the compounds inhibit an early step in the viral life cycle and that the crystal structure of human CycT1 fused with EIAV Tat is not suitable as a model for in silico screening. Although the amino acids of the target site were not completely identical between the previous study and this study, both of them included the TRM. Therefore, the addition of TAR RNA into the CycT1/Tat complex seems to indirectly alter the conformation of the TRM, and this alteration may be important to identify HIV-1 transcription inhibitors by in silico screening.

To define the docking pose of C3 with CycT1, we conducted docking studies of compounds and found a suitable docking pose of C3, where the in vitro anti-HIV-1 activity of the compounds was correlated with their in silico docking energy (Fig. 8). In fact, all of the inactive compounds (C4–C10 in Fig. 2) did not bind to CycT1 in silico (data not shown). The best docking pose revealed that C3 interacted with V199, L203, F241, K253, and R259 and was near the surface of Q172 and F176 of CycT1. Since Q172, F176, and R259 of human CycT1 interact with HIV-1 Tat, these amino acids appear to be particularly important for the anti-HIV-1 activity of C3. Consequently, this docking pose and structure-activity relationship of the series of compounds may provide useful information on design for more effective inhibitors.

In conclusion, the in silico screening system described herein is a useful tool for investigating novel inhibitors that target the TRM of human CycT1, and C3 identified in this system is considered to be a promising lead for novel HIV-1 transcription inhibitors.
Acknowledgements

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Figure legends

Fig. 1. Molecular model for in silico screening. The molecular surface of the target site in human CycT1 model is shown. (A) The target site for in silico screening determined by MOE software. The white and red spheres indicate the hydrophobic and hydrophilic properties, respectively. These spheres were used for docking of compounds. (B) The amino acids of CycT1 within 4.5 Å from the spheres in panel A. The amino acids interacting with Tat or TAR RNA were underlined. The target sites were shown in color, according to the amino acid properties. The red (D), blue (R and K), light green (F and W), green (A, L, and V), and magenta (T, Q, N, and S) indicate acidic, basic, aromatic, and neutral hydrophilic amino acids, respectively.

Fig. 2. Chemical structures of compounds. C1 and C2 were identified by in silico screening. C3–C10 were searched by PubChem (http://pubchem.ncbi.nlm.nih.gov/).

Fig. 3. Anti-HIV-1 activity of C1–C3 in chronically infected cells. OM-10.1 (A–C) and U1 (D) cells were incubated in the presence of various concentrations of the test compounds. After incubation for 24 h, the cells were stimulated with TNF-α (0.1 ng/ml) for 72 h. The p24 levels in the culture supernatants (line) and cell viability (column) were determined by ELISA and the MTT method, respectively. Data are expressed as the percent of controls (p24 level and viable cell number in the absence of compounds). All experiments were carried out in duplicate, and mean values are shown.

Fig. 4. Anti-HIV-1 activity of C3 in acutely infected cells. CEM (A) and MOLT-4 (B)
cells were infected with HIV-1 at a MOI of 0.001, and PBMCs (C) were infected with HIV-1 at a MOI of 0.01. The infected cells were incubated in the presence of various concentrations of the compound. After incubation for 72 h, the cells were subcultured and further incubated for 72 h. The p24 levels in the culture supernatants (line) and cell viability (column) were determined by ELISA and the MTT method, respectively. Data are expressed as the percent of control (p24 level and viable cell number in the absence of compounds). All experiments were carried out in duplicate, and mean values are shown.

**Fig. 5. Inhibitory effect of C3 on Tat-mediated transcription.** W-3 (A and B) and KM-3 (C and D) cells were treated with TNF-α (A and C) or transfected with the Tat-expression plasmid (B and D). The cells were cultured in the presence of various concentrations of the compound. After incubation for 24 h, the culture supernatants were collected and examined for their SEAP levels (line). The viable cell number (column) was determined by the MTT method. Data are expressed as the percent of control (SEAP level and viable cell number in the absence of compounds). All experiments were carried out in duplicate, and mean values are shown.

**Fig. 6. Inhibitory effects of C3 on Tat-induced phosphorylation of RNAPII.** W-3 cells were transfected with the Tat-expression plasmid and cultured in the presence of various concentrations of the compound. After incubation for 48 h, the whole cell lysates were subjected to Western blot analysis with anti-phosphorylated RNAPII (Ser2), anti-RNAPII, and anti-GAPDH antibodies. The panels A and B show the bands of blots and the amounts of phosphorylated RNAPII (Ser2P) (closed column) and total RNAPII.
(open column), both of which were normalized by the amount of GAPDH as a control. The levels of phosphorylated RNAPII and total RNAPII in W-3 cell without plasmid transfection are expressed as 100%. The experiments were performed twice, and the data represents means ± ranges for two separate experiments.

Fig. 7. Inhibitory effects of C3 on Tat-CycT1 interaction. CycT1 from CEM cell lysate was incubated with Tat in the presence of various concentrations of C3. After incubation for 2 h, the samples were subjected to Western blot analysis with anti-CycT1, anti-CDK9, and anti-Tat antibodies. (A) The left panel shows the samples precipitated with anti-CycT1 antibody- or goat IgG-immobilized beads in the absence of compound. The right panel shows the samples precipitated with anti-CycT1 antibody-immobilized beads in the absence or presence of the compound. (B) The amounts of CycT1 (closed circle), CDK9 (open circle), and Tat (column) were determined photodensitometrically, and those in the absence of the compound are expressed as 100%. The data represents means ± ranges for two separate experiments.

Fig. 8. Docking pose of C3 to CycT1. Docking pose 1 (A) and docking pose 2 (B) of C3 are indicated in the model structure of human CycT1. Three sites (Sites 1, 2, and 3) in the target region for in silico screening are shown in red, yellow, and blue, respectively. Docking energies of C1–C3 with human CycT1 in docking pose 1 (C) and docking pose 2 (D) were compared. Statistical significance was determined by Student’s t-test.
References


8. Butera ST, Perez VL, Wu BY, Nabel GJ, Folks TM. 1991. Oscillation of the human immunodeficiency virus surface receptor is regulated by the state of viral
activation in a CD4+ cell model of chronic infection. J. Virol. 65:4645-4653.


15. Garber ME, Wei P, KewalRamani VN, Mayall TP, Herrmann CH, Rice AP,
Littman DR, Jones KA. 1998. The interaction between HIV-1 Tat and human cyclin T1 requires zinc and a critical cysteine residue that is not conserved in the murine CycT1 protein. Genes Dev. 12:3512-3527.


Table 1

Anti-HIV-1 activity of C3 on chronically and acutely HIV-1-infected cells

<table>
<thead>
<tr>
<th>Cell</th>
<th>Infection</th>
<th>TNF-α</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt; (nM)</th>
<th>CC&lt;sub&gt;50&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt; (nM)</th>
<th>SI&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>OM-10.1</td>
<td>Chronic</td>
<td>+</td>
<td>617 ± 11</td>
<td>&gt; 10,000</td>
<td>&gt; 16</td>
</tr>
<tr>
<td>U1</td>
<td>Chronic</td>
<td>+</td>
<td>168 ± 97</td>
<td>&gt; 10,000</td>
<td>&gt; 60</td>
</tr>
<tr>
<td>CEM</td>
<td>Acute</td>
<td>-</td>
<td>19.9 ± 3.4</td>
<td>&gt; 1,000</td>
<td>&gt; 50</td>
</tr>
<tr>
<td>MOLT-4</td>
<td>Acute</td>
<td>-</td>
<td>17.9 ± 6.6</td>
<td>&gt; 1,000</td>
<td>&gt; 55</td>
</tr>
<tr>
<td>PBMCs</td>
<td>Acute</td>
<td>-</td>
<td>9.6</td>
<td>6,800</td>
<td>704</td>
</tr>
</tbody>
</table>

*<sup>a</sup> EC<sub>50</sub>, 50% effective concentration that inhibits the level of p24 antigen in culture supernatants by 50%.

*<sup>b</sup> CC<sub>50</sub>, 50% cytotoxic concentration that reduces the viability of TNF-α-untreated OM-10.1 cells and U1 cells or mock-infected CEM cells, MOLT-4 cells, and PBMCs by 50%.

*<sup>c</sup> SI, selectivity index based on the ration of CC<sub>50</sub> to EC<sub>50</sub>.

All data represents means ± standard deviations for three separate experiments.
Table 2.
Effect of C3 on TNF-α- or Tat-induced HIV-1 LTR transactivation

<table>
<thead>
<tr>
<th>Cell</th>
<th>Tat</th>
<th>TNF-α</th>
<th>EC₅₀ (nM)</th>
<th>CC₅₀ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W-3</td>
<td>+</td>
<td>−</td>
<td>183 ± 50</td>
<td>&gt; 2,000</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>+</td>
<td>&gt; 2,000</td>
<td>&gt; 2,000</td>
</tr>
<tr>
<td>KM-3</td>
<td>+</td>
<td>−</td>
<td>107 ± 11</td>
<td>&gt; 2,000</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>+</td>
<td>&gt; 2,000</td>
<td>&gt; 2,000</td>
</tr>
</tbody>
</table>

a IC₅₀, 50% inhibitory concentration that inhibits SEAP activity in culture supernatants by 50%.

b CC₅₀, 50% cytotoxic concentration that reduces the cell viability by 50%.

All data represents means ± standard deviations for three separate experiments.
Fig. 2
Fig. 3

A. C1 (OM-10.1)  
Viable cell number and p24 (%)  
Concentration (nM)

B. C2 (OM-10.1)  
Viable cell number and p24 (%)  
Concentration (nM)

C. C3 (OM-10.1)  
Viable cell number and p24 (%)  
Concentration (nM)

D. C3 (U1)  
Viable cell number and p24 (%)  
Concentration (nM)
Fig. 4

A

B

C

Viable cell number and p24 (%)

Concentration (nM)

Viable cell number and p24 (%)

Concentration (nM)

Viable cell number and p24 (%)

Concentration (nM)
Fig. 5

A

W-3/TNF-α

B

W-3/Tat

C

KM-3/TNF-α

D

KM-3/Tat

Viable cell number and SEAP activity (%)

Concentration (nM)

0 125 250 500 1000

0 125 250 500 1000
Fig. 6

A

<table>
<thead>
<tr>
<th>C3 (nM)</th>
<th>0</th>
<th>100</th>
<th>500</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tat</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RNAPII (S2P)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNAPII (total)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
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B

Percentage

<table>
<thead>
<tr>
<th>Tat</th>
<th>-</th>
<th>+</th>
<th>+</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>100</td>
<td>500</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3 (nM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>
Fig. 7

A

Immunoprecipitation

<table>
<thead>
<tr>
<th></th>
<th>CycT1</th>
<th>CDK9</th>
<th>Tat</th>
</tr>
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<tbody>
<tr>
<td>Anti-cycT1 IgG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3 (µM)</td>
<td>0</td>
<td>1</td>
<td>100</td>
</tr>
</tbody>
</table>

B

Percentage

<table>
<thead>
<tr>
<th>C3 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>100</td>
</tr>
</tbody>
</table>
Fig. 8

A Docking pose 1

B Docking pose 2

C Docking pose 1

D Docking pose 2

Leu203
Val199
Arg259
Phe176
Gln172
Phe241
Lys253
Site 1
Site 2
Site 3

Docking energy

C1 C2 C3

p=0.000001
p=0.2
p=0.000003

p=0.000008
p=0.02
p=0.0002

Docking energy

C1 C2 C3