Title

A small molecule, ACAi-028, with anti-HIV-1 activity targets a novel hydrophobic pocket on HIV-1 capsid

Running title: *Anti-HIV-1 agent targets novel pocket on HIV-1 capsid*

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19 **Abstract**

20 The human immunodeficiency virus type 1 (HIV-1) capsid (CA) is an essential viral component of
 21 HIV-1 infection, and an attractive therapeutic target for antivirals. We report that a small molecule,
 22 ACAi-028, inhibits HIV-1 replication by targeting a hydrophobic pocket in the N-terminal domain of
 23 CA (CA-NTD). ACAi-028 is one of more than 40 candidate anti-HIV-1 compounds identified by in
 24 silico screening and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. Our
 25 binding model showed that ACAi-028 interacts with the Q13, S16, and T19 amino acid residues, via
 26 hydrogen bonds, in the targeting pocket of CA-NTD. Using recombinant fusion methods, TZM-bl,
 27 time-of-addition, and colorimetric reverse transcriptase (RT) assays, the compound was found to exert
 28 anti-HIV-1 activity in the early stage between a reverse transcriptase inhibitor, azidothymidine (AZT),
 29 and an integrase inhibitor, raltegravir (RAL), without any effect on RT activity, suggesting that this
 30 compound may affect HIV-1 core disassembly (uncoating). Moreover, electrospray ionization mass
 31 spectrometry (ESI-MS) also showed that the compound binds directly and non-covalently to the CA
 32 monomer. CA multimerization and thermal stability assays showed that ACAi-028 decreased CA
 33 multimerization and thermal stability via S16 or T19 residues.

34 **Importance**

35 These results indicate that ACAi-028 is a novel CA inhibitor that binds to the novel hydrophobic
 36 pocket of CA-NTD. This study demonstrates that a compound targeting the new hydrophobic pocket
 37 is a promising anti-HIV-1 inhibitor. The findings presented here may offer the development of a novel

class of anti-viral agents that can be used, providing HIV-1 patients with more options for Anti-retroviral therapy (ART) treatment. Despite many years of successful pharmaceutical developments in the area of anti-retroviral therapy, the prevalence of drug-resistant mutations in HIV-1, necessitates the continued development of novel agents, such as ACAi-028.

Key words

HIV-1, anti-HIV-1 agent, HIV-1 capsid, *in silico* drug screening

Introduction

HIV-1 is a retrovirus that has affected humans for half a century, causing 700 000 deaths and 1.7 million new infections in 2019, according to the Joint United Nations Programme on HIV and AIDS (<https://www.unaids.org/en>). Significant progress has been made in recent years to understand HIV infection and design drugs to counteract multiple stages of the viral life cycle. Anti-retroviral therapy (ART) has largely allowed HIV-infected patients to have the same life expectancy as uninfected individuals (1, 2). Despite of the benefits of ART, HIV-1 often acquires drug-resistant mutations, (3) resulting in treatment failure. High adherence to ART is required to sustain viral suppression during HIV clinical treatment (4). It is especially important that we continue to discover new anti-HIV-1 agents that have potent antiviral activity with different mechanisms, providing HIV patients with more options for ART treatment.

57 The HIV-1 capsid protein (CA) plays an essential role in both the early and late stages of the HIV-1
58 life cycle. One of the key periods in the early stage is HIV-1 capsid core disassembly, also known as
59 uncoating (5, 6). After HIV-1 infection of the host cell via CD4 and CXCR4/CCR5 molecules,
60 uncoating occurs in a regulated manner. The exact mechanism of uncoating remains unclear. Previous
61 studies have suggested several hypotheses regarding the time and location of uncoating (7). Electron
62 microscopy results suggest that uncoating occurs near the surface of the plasma membrane (8), while
63 other studies have suggested that uncoating occurs in the cytoplasm, or tethered to nuclear import (9).
64 However, recent studies have indicated the possibility that uncoating actually occurs in the nucleus,
65 and might be coupled with both reverse transcription and integration (10, 11, 12). A new inhibitor of
66 CA uncoating has the potential to provide novel insights and tools for further research in this field.

67 Representative anti-CA compounds such as CAP-1 (13, 14), PF-3450074 (PF74) (15, 16), ebselen
68 (17), BD-1, BM-1 (18), I-XW-053 (19), and C1 (20) have been discovered, although none have been
69 approved for clinical use by the FDA. In this study, we used PF74 and ebselen as control drugs. PF74
70 is a lead compound of GS-CA1 (21) and lenacapavir, formerly known as GS-6207, (22, 23) that have
71 been developed by Gilead Sciences, Inc. These compounds interact with the CTD-NTD interface and
72 hinges between CA monomers, inducing hyperstabilization of the HIV-1 core and inhibiting the
73 interaction of co-factors, such as Nup153 and CPSF6 (16). Lenacapavir has advanced to phase 2/3 of
74 the clinical CAPELLA trial (ClinicalTrials.gov Identifier: NCT04150068), and preliminary reports
75 from this trial indicate that it is effective at reducing the viral load of patients on failing treatment

regimens, strongly suggesting that the CA is a viable target for the development of novel anti-HIV-1 compounds. Ebselen is also a unique CA inhibitor that covalently binds to the C-terminal domain of CA via Cysteine residues, inhibiting HIV-1 activity (17).

We previously reported that the insertion of a short amino acid sequence near the Arg18/Thr19 region of decreased its stability, inducing abnormal CA degradation (24), and identified an adequate hydrophobic cavity near this region on the surface of CA-NTD, which could be a potential drug target. To the best of our knowledge, none of the other published anti-CA compounds are known to target this hydrophobic cavity.

In this study, we searched for new compounds capable of interacting with the hydrophobic pocket from a library containing millions of commercially available compounds via *in silico* docking simulations. We identified several compounds as candidate HIV-1 inhibitors that were capable of preventing HIV-1 replication. Here, we highlight ACAi-028, a CA inhibitor with unique molecular characteristics, which possess potent anti-HIV-1 activity. It is anticipated that both this compound and the identified binding pocket hold potential therapeutic and research applications.

Results

Identification of candidate compounds that target a novel hydrophobic cavity of the HIV-1 capsid

94 CA protein consists of the N-terminal domain (CA-NTD, amino acid residues 1-145) and the C-
 95 terminal domain (CA-CTD, residues 151-231) linked via a short flexible region (residues 149-150)
 96 (25, 26, 27). CA-NTD comprises one β -hairpin, seven α -helices, and a cyclophilin binding loop
 97 (CypA-BL), while CA-CTD has a 3_{10} -helix and four α -helices, as shown in Fig. 1A. Recently, CA
 98 inhibitors have attracted attention due to the development of lenacapavir, whose lead compound is
 99 PF74 (15), which exhibits a long-acting, strong anti-HIV profile. We have reported that the insertion
 100 of a short amino acid sequence into the CA-NTD, specifically near the R18 and T19 residues, results
 101 in spontaneous CA degradation (24).

102 To identify new capsid inhibitors, we selected a novel hydrophobic pocket (Fig. 1A) near this position
 103 as a potential interaction site for drug candidates with the CA-NTD. *In silico* docking simulations were
 104 performed to search for new compounds that interact with this pocket from a database of over eight
 105 million commercially available compounds. The selection process is illustrated in Figure 1B. More
 106 than 40 compounds were identified as candidate HIV-1 inhibitors that prevented HIV-1_{LAI} replication,
 107 using the MTT assay.

108 In this study, we identified ACAi-028 (Fig. 1C), which has a small molecular weight (MW) of 381
 109 g/mol, and potent anti-HIV-1_{LAI} activity (EC₅₀, 0.55 μ M), among the candidate HIV-1 inhibitors.
 110 Therefore, we examined the anti-HIV profile of ACAi-028.

111 The crystal structure of CA-NTD_{1-146/Δ87-99G} was produced (15) (Fig. S1), and the binding profile of
 112 ACAi-028 to the CA-NTD was elucidated using a docking model (Fig. 1D). The binding profile of

ACAi-028 with the CA-NTD is similar to that of ACAi-028 with full-length CA (Protein Data Bank [PDB] accession number 4XFX) (28) (Fig. S2). ACAi-028 is estimated to form two hydrogen bonding (H-bond) interactions with the side-chains of Q13 and T19 (inter-atomic distances of 1.87 and 1.89 Å, respectively), and one H-bond interaction with the main chain of S16 (inter-atomic distance of 2.06 Å). This structural arrangement suggests that ACAi-028 could potentially bind to the newly identified hydrophobic cavity in CA-NTD.

ACAi-028 inhibits the early stage of the HIV-1 life cycle

Next, we determined the anti-HIV activity (EC₅₀s) of ACAi-028 against various HIV-1 and HIV-2 strains, in comparison with several compounds that have been previously reported as capsid inhibitors, such as PF74 (15) and ebiselen (17), and an RT inhibitor, AZT (29) (Table 1).

ACAi-028 exerted potent anti-HIV-1 activity against single-round HIV-1 infection using VSV-G pseudo-typed HIV-1_{NL4-3} (HIV-1_{VSV-G dENV}), suggesting that ACAi-028 inhibits the early stage of the HIV-1 life cycle. ACAi-028 also prevented multiple-round HIV-1 infection using HIV-1 strains (HIV-1_{NL4-3} and HIV-1_{LAI}), except for HIV-2 strain (HIV-2_{ROD}), at a concentration similar to that of PF74, ebiselen, and AZT. In addition, ACAi-028 inhibited the replication of various types of HIV-1 strains (HIV-1_{104pre}, HIV-1_{MDR/B}) (30) in peripheral blood mononuclear cells (PBMCs) and HIV-1_{ATV^R}_{5μM} in MT-4 cells (Table 2), and had negligible cytotoxicity in all the cell lines we tested (Table 3).

To examine the details of early stage inhibition by ACAi-028, we performed fusion (31), TZM-bl, time-of-addition (32), and colorimetric RT activity assays. ACAi-028 did not significantly inhibit the entry step of HIV-1_{LAI}, in comparison to the entry inhibitor, AMD3100 (33) (Fig. 2A). ACAi-028 also displayed strong anti-HIV-1 activity against HIV-1 R5 strains, including HIV-1_{Ba-L}, and HIV-1_{JR-FL}, and X4 strains, except for HIV-2_{ROD}, using the TZM-bl assay (Fig. 2B).

A time-of-addition assay was conducted to compare the inhibition time of ACAi-028 with that of the other classes of anti-HIV-1 drugs. As expected, AMD3100 ceased displaying anti-HIV-1 activity when it was added more than 2 h post-infection, while an integrase inhibitor, RAL, significantly prevented HIV-1 infection beyond 10 h post-infection (Fig. 2C).

We observed that ACAi-028 inhibited anti-HIV-1 activity at a similar time as RT inhibitors, AZT and efavirenz (EFV) (34), as well as a CA inhibitor, PF74. Fifty percent inhibition induced by these drugs occurred between 4 and 8 h post-infection, which is consistent with a previous report (15). Moreover, we examined whether ACAi-028 prevents HIV-1 RT activity using a colorimetric RT activity assay and found that ACAi-028 and PF74 did not have a significant impact on HIV-1 RT activity, in comparison to EFV (Fig. 2D) *in vitro*. This suggests that ACAi-028 is unlikely to be an RT inhibitor. Taken together these results indicate that ACAi-028 may have a similar level of potency as a CA inhibitor as PF74, which affects the CA uncoating process.

ACAi-028 does not affect the late stage of the HIV-1 life cycle

150 To examine the effect of ACAi-028 on late-stage inhibition, we examined whether ACAi-028
151 affects the process of HIV-1 production, including Gag proteolytic processing and maturation. Forty-
152 eight hours after transfection of pHIV-1_{NL4-3} into 293T cells in the presence of ACAi-028 (10 μ M),
153 PF74 (10 μ M), ebselen (10 μ M) or, a protease inhibitor, darunavir (DRV) (2 μ M) (35). Gag proteins
154 within the cells were observed by western blotting, and viral production was evaluated comparison to
155 p24 expression levels in the culture supernatant. ACAi-028, PF74, and ebselen did not affect Gag
156 proteins in the cells, unlike DRV (Fig. 3A), whereas ACAi-028 (88.7%), and ebselen (75.2%), PF74
157 (62.9%), and DRV (15.7%) reduced HIV-1 production, which is consistent with previous reports (15,
158 17) (Fig. 3B). Additionally, the effect of ACAi-028 on Gag proteolytic processing and maturation of
159 HIV-1 virions was investigated using western blotting and the TZM-bl assay. ACAi-028 did not affect
160 HIV-1 maturation, as observed via western blotting, using anti-Gag or anti-IN antibodies as well as a
161 DMSO control (Fig. 3C). ACAi-028 did not reduce the viable virions (111.8%), as opposed to DRV
162 (3.6%) (Fig. 3D), suggesting that ACAi-028 does not have any effect on HIV-1 production and
163 maturation. On the other hand, a high concentration of PF74 (10 μ M) did not affect Gag proteins in
164 the cell lysate (Fig. 3A), but the production level of virions in the presence of PF74 significantly
165 decreased by 62.9% compared to that of the DMSO control. Furthermore, the infectivity of the virions
166 was reduced to 6.9% (Fig. 3B and D), suggesting that PF74 affects both the early and late stages, in
167 agreement with previous reports (15). It has been reported that ebselen does not exhibit late-stage
168 inhibition (17). Interestingly, premature products such as Gag-pol (p160), Gag-pol intermediate (p120),

Gag (p55), and Gag intermediates were found in the virion lysate that was produced at a high concentration (10 μ M) of Ebselen, via the western blotting with anti-Gag or anti-IN antibodies, which is similar to DRV (Fig. 3C). Additionally, the infectivity of the virions produced in the presence of ebselen (10 μ M) was significantly reduced by 64.6 % of their normal level (Fig. 3D). These results suggest that ebselen may interfere with HIV-1 maturation. These results indicate that ACAi-028 is unlikely to affect the late stage of the HIV-1 life cycle.

Conformational difference of targeting the hydrophobic pocket between HIV-1 and HIV-2

ACAi-028 did not show anti-HIV activity against the HIV-2_{ROD} strain (Table 1). We compared the amino acid sequences of experimental HIV-1 strains, such as HIV-1_{NL4-3}, HIV-1_{HXB (LAI)}, HIV-1_{Ba-L}, and HIV-1_{JR-FL}, which are all closely related to simian immunodeficiency virus from chimpanzees (SIV cpz). Similarly, comparisons of experimental HIV-2 strains, such as HIV-2_{ROD} and HIV-2_{EHO}, which are related to SIV sooty mangabeys (SIV smn), as shown in Fig. 4A, were also undertaken. The Q13, S16, and T19 residues, which are expected to play important roles in ACAi-028 binding to CA (Fig. 1D), are conserved among all HIV-1 strains and SIV cpz, whereas most of the amino acids from residues 2 to 15 of HIV-2 are different from those of HIV-1 (Fig. 4A). Moreover, Q13, S16, and T19 residues were highly conserved across 6144 sequences from all HIV-1 subtypes (HIV sequence

database filtered web alignment) at conservation rates of 99.89%, 97.85%, and 99.72 %, respectively (Fig. 4B).

The crystal structures of the ACAi-028 target hydrophobic pocket of HIV-1_{NL4-3} ([PDB] accession number 4XFX) (28) and HIV-2_{ROD} ([PDB] accession number 2WLV) (36) are shown in Fig. 4C. This HIV-1_{NL4-3} pocket seems to have a sufficient volume for ACAi-028 binding, while that of HIV-2_{ROD} appears to be too shallow for binding (Fig. 4C and Fig. S3). The cavity of CA-NTD_{NL4-3} is covered by that of the CA-NTD_{ROD} in the overlay of these crystal structures, as shown in Fig. 4C, suggesting that the ACAi-028 target volume of HIV-2 is clearly smaller than that of HIV-1.

Moreover, we examined the binding ability of ACAi-028 to CA-NTD_{ROD} using a binding model. As shown in Fig. 4D, there were no bridging H-bonds between ACAi-028 and the amino acid residues of CA-NTD_{ROD}, corresponding to the lack of inhibition ($EC_{50} > 10 \mu M$) of ACAi-028 against HIV-2_{ROD} (Table 1). These results indicate that ACAi-028 may fail to interact with HIV-2_{ROD} CA, resulting in no anti-HIV-2 activity.

Binding profiles of ACAi-028 to CA proteins.

In order to observe the direct binding of ACAi-028 to CA, we produced recombinant HIV-1_{NL4-3}-derived CA proteins using *E. coli*, and examined the binding of ACAi-028 to these proteins using an electrospray ionization mass spectrometry (ESI-MS) (37). The ESI-MS spectra of the CA monomer with 1% methanol revealed nine peaks of charged ions in the range of mass/charge ratio (m/z) range

of 1,100–1,900 (Fig. 5A). The MW estimated from the peaks of charged ions (deconvoluted ESI-MS spectrum) was 25601.9 Da, corresponding to the theoretical MW of intact CA monomer (25602.5 Da), as calculated by Peptide Mass Calculator v3.2 (Fig. 5A). After treatment of CA with ACAi-028, peaks associated with ACAi-028 binding to CA emerged in the m/z range of 1,300–1,900 next to each spectrum of the CA monomer (Fig. 5B). The deconvoluted ESI-MS spectrum revealed a peak associated with CA and ACAi-028 at 25984.1 Da, which was similar to the sum of the MW of a CA monomer (25601.9 Da) and ACAi-028 (381 Da) (Fig. 5B). Additionally, we examined whether ACAi-028 binds covalently to CA. After treatment of CA with ACAi-028 or ebselen, the samples were denatured by acetonitrile and trifluoroacetic acid. The binding peak of CA with ACAi-028 was not detected in Fig. 5C, while two strong peaks of CA with ebselen (25922.03 and 26120.59 Da, representing CA combined with one or two ebselen molecules, respectively) were observed (Fig. 5D). These results are in line with a previous report (17), suggesting that ACAi-028 binds non-covalently to CA, unlike ebselen. Thus, ACAi-028 binds directly and non-covalently to the CA monomer.

ACAi-028 affects the molecular characterization of CA proteins via S16 and T19 residues.

ACAi-028 was shown to interact directly with the CA proteins using ESI-MS. To investigate how ACAi-028 interacts with CA proteins, we produced CA variants carrying S16E (CA_{S16E}) or T19A (CA_{T19A}) amino acid substitutions, which were intended to alter the binding ability of ACAi-028 to CA.

225 CA_{M185A}, carrying an M185A amino acid substitution was also produced as previously reported (38),
226 as a control.

227 CA thermal stability in the presence of ACAi-028 was examined using differential scanning
228 fluorimetry (DSF) (39, 40) (Fig. 6). The melting temperature (T_m) value for proteins generally
229 indicates the temperature at which the protein is unfolded by 50% folded (T_m 50). T_m 50 of wild-type
230 CA (CA_{WT}) increased in the presence of PF74, while that of ACAi-028 clearly decreased by 6.8°C and
231 7.1°C at 10 and 50 μM, respectively (Fig. 6A and B). T_m 50 of CA_{S16E} showed a mild reduction of
232 3.9°C at 50 μM, while that of CA_{T19A} was seen to remain unchanged in the presence of ACAi-028 (Fig.
233 6C and D), suggesting that S16 and T19 residues may be associated with the binding of ACAi-028 to
234 the target pocket of CA-NTD. Additionally, a CA multimerization assay was performed in the presence
235 of ACAi-028. The treatment of CA_{WT} with PF74 increased CA multimerization as previously
236 described (15), whereas ACAi-028 decreased CA_{WT} multimerization at 4 and 40 μM in a
237 concentration-dependent manner (Fig. 7A). M185A greatly decreased the CA multimerization, in
238 agreement with, a previous report (38). The addition of S16E substitution to CA proteins slightly
239 reduced CA_{S16E} multimerization, while T19A slightly increased CA_{T19A} multimerization in comparison
240 to CA_{WT} (Fig. 7B) at the same sodium concentration, suggesting that these residues significantly affect
241 the CA multimerization. In the presence of ACAi-028, CA_{S16E} multimerization was similar to that of
242 CA_{WT} (Fig. 7C). However, CA_{T19A} multimerization was not affected by the presence of ACAi-028,
243 even at a higher concentration (40 μM) (Fig. 7D), suggesting that the T19A mutant may inhibit ACAi-

028 binding to the target pocket under these conditions. These results strongly indicate that ACAi-028 may interact with CA proteins via S16 and T19 residues in the target pocket of CA-NTD.

ACAi-028 potentially interacts with the binding pockets of CA in the hexameric state.

ACAi-028 exerted anti-HIV-1 activity in the early stages of the HIV-1 life cycle. In the early stage, CA proteins constitute a capsid lattice (HIV core), which is composed of approximately 250 hexamers and exactly 12 pentamers (41). To confirm the location and space of the binding pocket in a CA hexamer, we analyzed a CA hexamer model in complex with, or without, ACAi-028, based on CA crystal structures ([PDB] accession number 3H4E (42), and 5MCX (32)). In the model, the ACAi-028 can bind to the pocket located at inside the CA hexamer ([PDB], 3H4E) (Fig. 8A). Additionally, the ACAi-028 binding profile to the pocket in a CA dimer extract from the CA hexameric state ([PDB], 5MCX) was predicted using our docking model. ACAi-028 can putatively interact with the pocket in the CA dimer of the CA hexameric state, without affecting the paired CA monomer, via two H-bonds with L43 and E45 (Fig. 8B). This suggests that ACAi-028 can potentially interact with the binding pockets of the CA hexameric state, in the HIV-1 core. Taken together, ACAi-028 is a novel capsid inhibitor that binds to the new hydrophobic pocket in CA-NTD, thereby inhibiting the early stage of HIV-1 replication.

Discussion

Based on the results described above, we concluded that ACAi-028 is a small molecular CA inhibitor of HIV-1 that interacts with CA via the novel region, which has not been previously reported (Fig. 9 and Table 4). The ACAi-028 binding pocket is formed by key residues, namely at Gln13, Ser16, and Thr19, which constitute the β -hairpin end, flexible linker, and front edge of α -helix 1 (Fig.1). To understand the mechanisms of action of CA inhibitors previously described (43), we categorized several candidate CA inhibitors based on their molecular characterization into groups A, B, C, and D (Fig. 9 and Table 4). CAP-1 (group A) is a late-stage inhibitor, without early stage inhibition of the HIV-1 life cycle (13, 14), possibly because the hexameric association of CA proteins in the mature HIV-1 core requires tight molecular packing, prevent access of CAP-1 to this pocket. Indeed, the other small molecules, including BD-1 and BM-1 (18), which share the CAP-1 binding region, are all late-stage inhibitors. Therefore, the inhibitory mechanism of group A is likely different from that of ACAi-028. PF74 (group B) interacts with a region distinct from ACAi-028 and CAP-1, as shown in Fig. 9 and Table 4, and binds to the pocket formed between CA-NTD and the CA-CTD of an adjacent CA monomer. PF74 is known to inhibit both the early and late stages of the HIV-1 life cycle (15), whereas ACAi-028 only inhibited the early stage (Fig. 2 and 3). As shown in the CA multimerization and DSF assay (Fig. 5), ACAi-028 has a significant effect on the CA multimerization and thermal stability, which are completely opposite to PF74, suggesting that ACAi-028 and PF74 represent different classes of CA inhibitors (Fig. 9 and Table 4). I-XW-053 (19) (group C) binds to, and disrupts CA NTD-NTD

interactions in CA hexamers during the early stages of HIV-1 infection. By SPR analysis of I-XW-053 binding with CA mutants, the proposed binding sites of I-XW-053 were found to involve I37 and R173, which the amino acids identified within the target binding region of ACAi-028. Group C includes C1 (20), a late-stage assembly inhibitor that interacts with the CA-NTD residues at E98, H120, and I124 residues. Ebselen (group D) is a covalent inhibitor of HIV-1 CA by forming a selenosulphide bond with C198 and C218 residues in the CA-CTD. ACAi-028 possesses distinct properties, in comparison to the inhibitors of groups C and D, suggesting that ACAi-028 targeting the hydrophobic pocket is likely to be different from any previously discovered CA inhibitors.

Amino acid substitutions that alter CA multimerization have been previously reported (38). Substitution of amino acid residues at S16 and T19 altered the CA multimerization; S16E mutants decreased the CA multimerization, whereas T19A mutants increased the CA multimerization. Additionally, E45A substitution, E45 residue is a residue predicted to be located in the ACAi-028-binding pocket, is known to increase the CA multimerization (45). These amino acids might constitute an important site for CA dimerization in the formation of CA hexamers (Fig. 8). The binding of ACAi-028 to the pocket could potentially interfere with CA dimerization, because this pocket is located within an inward facing portion of the CA hexamer (Fig. 8A), which is supported by the reduction of CA multimerization in the presence of ACAi-028 (Fig. 5A). To investigate the binding profile of ACAi-028 to CA-NTD, we produced crystals of CA-NTD_{1-146/Δ87-99G} proteins, according to a previous report (15), and utilized this structure for the preparation of a docking model for ACAi-028 binding to

301 CA. Unfortunately, when crystals of CA-NTD_{1-146/Δ87-99G} were produced in the presence of ACAi-028,
302 significant precipitations occurred without the emergency of a crystal in the droplet, suggesting that
303 ACAi-028 may cause CA proteins instability or destabilization of the CA protein. Thus, we were
304 unable to acquire actual binding profiles of ACAi-028 to CA. Moreover, when we have selected HIV-
305 1 variants resistant to ACAi-028, with increasing concentrations of up to 20 μM, we did not detect
306 ACAi-028-related strong resistant mutations in the CA (Gag) regions, suggesting that ACAi-028 might
307 still possess an unknown mechanism for HIV-1 inhibition. This represents a limitation of the present
308 study, and requires further investigation.

309 Recently, lenacapavir was reported to be a powerful anti-HIV compound, with broad-spectrum
310 inhibition, even against multidrug-resistant HIV-1, HIV-2, and SIV (21), for long-acting HIV-1
311 treatment (21, 22) (Phase 2/3 CAPELLA trial). Furthermore, lenacapavir has also been predicted to be
312 effective in HIV prevention as HIV pre-exposure prophylaxis (PrEP). These findings strongly suggest
313 that CA is an attractive therapeutic target for the development of novel antivirals.

314 In conclusion, we have identified ACAi-028 as a small molecular anti-HIV-1 CA inhibitor that
315 targets a novel hydrophobic CA-NTD pocket, exerting the early-stage inhibition of the HIV-1 life
316 cycle with EC₅₀ of 0.55 μM. Further research is underway to understand the role of this region in HIV-
317 1 replication. The novel hydrophobic pocket identified here, should be a viable target for the
318 development of new synthetic CA inhibitors. Furthermore, ACAi-028, a potent CA inhibitor targeting
319 this novel pocket, could have valuable therapeutic and research applications.

Materials and Methods

Cells and Viruses

MT-2 and MT-4 cells (Japanese Collection of Research Bioresources Cell Bank; JCRB Cell Bank, Japan) were cultured in RPMI1640 medium (Gibco, Thermo Fisher Scientific, USA) with Fetal Bovine Serum (FBS, Gibco, Thermo Fisher Scientific, USA), Penicillin (P), and Kanamycin (K). 293T, Li 7, HLE, and COS7 cells obtained from JCRB Cell bank and TZM-bl cells obtained from the NIH AIDS Research and Reference Reagent Program were cultured in low glucose DMEM with L-Glutamine and Phenol Red (Fujifilm Wako Pure Chemical Corporation, Japan), as well as FBS, P, and K. PHA-PBMCs were derived from a single donor in each independent experiment. The research protocols described in the present study were carried out in accordance with relevant guidelines and regulations, and were approved in Ethics Committee for Epidemiological and General Research at the Faculty of Life Sciences, Kumamoto University. The HIV-1 strains used in our experiments have already been established previously, including HIV-1_{NL4-3}, HIV-1_{LAI}, HIV-1_{Ba-L}, HIV-1_{JR-FL}, HIV-1_{ERS104pre} which was isolated from clinical HIV-1 strains of drug-naïve patients with AIDS (46), and HIV-1_{MDR/B} which was originally isolated from AIDS patient who had received 9 anti-HIV-1 drugs over the 34 months and was genotypically and phenotypically characterized as multi-drug-resistant HIV-1 variant (47, 48). HIV-1_{VSV-G dENV} was produced by co-transfection of pCMV-VSV-G vector (addgene) and pNL4-3 dENV with deleted Kpn1-Nhe1 site in the Env region into 293T cells.

Plasmid constructs

Full-length CA sequences derived from pNL₄₋₃ was introduced to pET30a vectors (Novagen-Merck KGaA, Germany), producing a pET30a CA vector. The site-directed mutagenesis was performed using PrimeSTAR[®] Max (Takara Bio Inc. Japan) to introduce S16E, T19A, E45A, and M185A into the pET30a CA, producing pET30a CA_{S16E}, CA_{T19A}, and CA_{M185A} vectors, respectively. CA-NTD₁₄₆ deletion sequence carrying a single glycine residue instead of CypA-BL (residues 87–99) (the amino acids sequence is based on (15)) was introduced to pET30a vectors 6xHis at N-terminus, producing a pET30a His-CA_{1-146/Δ87-99G} vector

Protein expression and purification

CA proteins were produced from the pET30aCA in *E. coli* Rosetta[™] (DE3) pLysS Competent Cells (Novagen) grown in LB medium supplemented with Kanamycin and chloramphenicol at 37°C, and induced with 1.0 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 3-5 hr at 37°C. The bacterial cells were harvested and stored at -80°C. The pellets of CA were resuspended and sonicated in CA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM 2-mercaptoethanol (BME)) supplemented with 0.5 mM phenylmethylsulfonylfluoride. The lysates were cleared by centrifugation for 15 min at 3,500 rpm at 4°C. After 5M NaCl was added to the supernatants, the samples were cleared by centrifugation for 15 min at 3,500 rpm again. The precipitate was resuspended in the CA buffer. The sample was precleared for 15 min at 15,000 rpm at 4°C, and the supernatants were filtered through a 0.45 μm filter.

The CA proteins were loaded onto a HiLoad 16/60 Superdex 200 column (GE Healthcare, USA), and were eluted with CA loading buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM BME) using AKTAprime plus (GE Healthcare). CA monomer fractions were concentrated using Amicon Ultra-10K device (Merck Millipore) in the CA buffer. The proteins concentration was determined using a BCA Protein Assay Reagent Kit (Thermo Fisher Scientific) and stored at -80°C.

***In silico* simulation and docking model**

The crystal structures of CA-NTD_{1-146/Δ87-99G} produced by our method and various full-length CA proteins ([PDB] accession number 4XFX, 3H4E, and 5MCX) from the RCSB Protein Data Bank (<http://www.rcsb.org>) are utilized for the docking simulation. Hydrogens were added to 2D structure of ACAi-028, and the structures were energy-minimized with the MMFF94x force field as implemented in MOE (Chemical Computing Group, Quebec, Canada). All docking simulations were performed with SeeSAR and FlexX version 10 (BioSolveIT GmbH, Sankt Augustin, Germany). Molecular graphics and analysis were performed with UCSF Chimera (<https://www.rbvi.ucsf.edu/chimera>).

Fusion assay

Fusion assay was performed previously (31). In brief, 293T cells were transfected with pHIV-1_{NL4-3} Tat with or without pHIV-1_{NL4-3} Env while COS-7 cells were transfected with CD4, CXCR4, and LTR-

Luciferase. After 24 hr of incubation at 37°C and 5% CO₂, the transfected 293T cells were mixed with the transfected COS-7 cells in the presence or absence of tested compounds for 6 hr. The luciferase of the samples was detected using Firefly Luciferase Reporter Assay Kit I (PromoCell GmbH, Germany) and the luciferase intensity was normalized to the negative control. Ratios of luciferase intensity of the samples were compared.

Time-of-addition assay

Time-of addition assay is previously reported (32). Briefly, TZM-bl cells (5×10^4 /mL) were subjected to HIV-1_{LAI} (50 ng/mL of p24) in 96-well white plates. Drugs at the indicated concentrations were also added to the set of wells demarcated for 0 hr. After 2 hr of incubation at 37°C and 5% CO₂, The supernatant was removed and the cells were washed once to remove all traces of the virus. Drugs at the respective concentration were added back to the cells marked for 0 hr as well as the ones marked for 2 hr. Subsequently, every 2 hr after, drugs at the correct concentration was added to the next each well until 10 h after incubation. The cells were incubated at 37°C and 5% CO₂ until 48 hr. Luciferase intensity was measured using FluoSTAR Omega (BMG labtech GmbH, Germany).

TZM-bl assay

TZM-bl cells were obtained from the NIH AIDS Research and Reference Reagent Program. TZM-bl assay was performed in 96-well white plate using the Firefly Luciferase Reporter Assay Kit I. Briefly,

the supernatant medium was removed and lysis buffer added to the samples. After the samples were shaken for 25 minutes, D-luciferin was added to each well. After shaking, the luciferase intensity was measured using FluoSTAR Omega.

Virus quantification

Virus samples were measured by an HIV-1 p24 enzyme-linked immunosorbent assay (ELISA) using Lumipulse G1200 (Fujirebio Inc, Japan), and normalized to determine the viral concentration.

Reverse Transcriptase Assay

Colorimetric reverse transcriptase assay was performed (Reverse Transcriptase Assay colorimetric, Roche, Switzerland). Briefly, recombinant HIV-1 reverse transcriptase was added to tested compounds dissolved in the lysis buffer and subsequently incubated at 37°C and 5% CO₂ for 3-4 hr. After washing, peroxidase-conjugated anti-digoxigenin antibody solution were added to the samples and incubated for 1 hr at 37°C and 5% CO₂. After washing, peroxidase substrate ABTS solution with enhancer was added to the samples. The optical density was measured at 405 nm using a Versamax microplate reader (Molecular Devices, USA).

Western blotting

Western blotting was described previously (40). Briefly 293T cells were plated and incubated for 24

hr at 37°C in 5% CO₂. Cells were transfected with pNL₄₋₃ vectors using Attractene Transfection Reagent (QIAGEN, Germany). After 8 hr, the medium was changed and the tested compounds were added, and then incubated for 48 hr. Subsequently, the viruses were filtered, purified by ultracentrifugation in 15% sucrose–phosphate-buffered saline (PBS), normalized by the p24 levels, and stored in PBS at -80°C. The cells were lysed in M-per buffer (Thermo Fisher Scientific) supplemented with Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific). The samples were titrated using BCA Protein Assay Kit and stored at -80°C. The samples were prepared and separated with SDS-PAGE (5-20% Extra PAGE One Precast Gel; nacalai tesque) and transferred onto a nitrocellulose membrane. The samples were detected with anti-HIV-1 Gag (p55 + p24 + p17) antibody (catalog number ab63917; Abcam), anti-HIV-1 IN antibody (catalog number ab66645; Abcam), second mouse or rabbit antibody (MBL co., LTD.), and anti-beta actin antibody (HRP conjugated) (Abcam), and then visualized using SuperSignal WestPico Chemiluminescent Substrates (Thermo Fisher Scientific).

CA multimerization assay

CA multimerisation assays were performed as previously reported (38). Compound was added to 30 µM of CA protein in 50 mM phosphate buffer at pH 8.0 supplemented with 50mM NaCl. Capsid assembly was initiated by addition of 50 mM sodium phosphate at pH 8.0 supplemented with 5M NaCl. Optical density at 350 nm was measured using FLUOstar Omega (BMG labtech) for 1 hr.

434

435 **DSF**

436 DSF method was previously described (39, 40). In brief, recombinant CA proteins (25 μ M) were
 437 prepared in PBS. After CA treatment with tested compounds for 5-8 hr on RT, SYPRO Orange (Life
 438 Technologies) was added to the samples (final concentration of SYPRO orange: 5 $\times$). The samples
 439 were successively heated from 25 to 95°C, and the increasing fluorescence intensities were measured
 440 by the real-time PCR system 7500 Fast (Applied Biosystems, Thermo Fisher Scientific). Data
 441 indicated is as a relative ratio between minimum and maximum intensity of SYPRO orange from 25
 442 to 95°C detected for each sample.

443

444 **ESI-MS**

445 ESI-MS protocol was previously described (37). In brief, MS spectra of CA in the presence of ACAi-
 446 028 were obtained using an electrospray ionization (ESI) quadrupole time-of-flight (QTOF) mass
 447 spectrometer (impact II, Bruker Daltonics). Each sample solution in native condition was introduced
 448 to the ESI-QTOF mass spectrometer through an infusion pump at a flow rate of 3.3 μ L/min. To detect
 449 the denatured samples, analysis was done using the QTOF mass spectrometer equipped with a Captive
 450 Spray electrospray ionization platform with liquid chromatography (Ultimate 3000 HPLC, Thermo
 451 Fisher scientific). The following ion source parameters have been applied: dry Heater: 150°C, dry Gas:
 452 8.0 L/min, capillary voltage: 1000 V, End plate offset: -500 V. MS scans have been acquired at a

spectra rate of 1 Hz at a mass range from 100 to 3000 m/z. Molecular weights by protein deconvolution were determined using Data Analysis 4.4 (Bruker Daltonics). M.W. of CA proteins was calculated using Peptide Mass Calculator v3.2 (<http://rna.rega.kuleuven.be/masspec/pepcalc.htm>)

Crystallization and X-ray data collection

The crystallization procedure was performed according to methods in a previous report (15) ([PDB] 2XDE). Briefly, crystallization was performed by the hanging-drop vapor diffusion method using EasyXtal 15-well tools (QIAGEN). CA-NTD_{1-146/Δ87-99G} proteins were expressed and purified as described above. The protein concentration was adjusted to 2 mg/ml. The reservoir solution consists of 100 mM phosphate-citrate pH 4.2, 200 mM NaCl, and 20% PEG 8000. The crystals reached 0.2 to 0.4 mm within 1 week at 10°C. The crystals were transferred to a reservoir solution supplemented with 25% glycerol and flash-frozen at 100 K. Then, X-ray diffraction experiments were carried out. Data collection and refinement statistics were subsequently examined.

Drug Susceptibility Assay

The susceptibility of HIV-1_{LAI} and HIV-2_{ROD} to ACAi-028 and control drugs/compounds were determined as previously described (49). Briefly, MT-2 cells (10⁴/ml) were exposed to 100 × 50 % tissue culture infectious dose (TCID₅₀) of HIV-1_{LAI} or HIV-2_{ROD} in the presence or absence of various

471 concentrations of compounds in 96-well plates and were incubated at 37°C for 7 days. After incubation,
 472 100 µl of the medium was removed from each well, 3-(4,5-dimethylthiazol-2-yl)-2,5-
 473 diphenyltetrazolium bromide (MTT) solution was added to each well in the plate, followed by
 474 incubation at 37°C for 1.5-4 hr. After incubation to dissolve the formazan crystals, acidified
 475 isopropanol containing 4% (v/v) Triton X-100 was added to each well and the optical density measured
 476 in a kinetic microplate reader (Vmax; Molecular Devices, Sunnyvale, CA). All assays were performed
 477 in duplicate. 50% cytotoxic concentration (CC₅₀) of compounds/drugs for each cell line was also
 478 evaluated by MTT assay. To determine the sensitivity of primary HIV-1 isolates to compounds, PHA-
 479 PBMC (10⁶/ml) were exposed to 50 TCID₅₀ of each primary HIV-1 isolate and cultured in the presence
 480 or absence of various concentrations of drugs in 10-fold serial dilutions in 96-well plates. In
 481 determining the drug susceptibility of certain laboratory HIV-1 strains, MT-4 cells were employed as
 482 target cells as previously described (50, 51) with minor modifications. In brief, MT-4 cells (10⁵/ml)
 483 were exposed to 100 TCID₅₀ of drug-resistant HIV-1 strains in the presence or absence of various
 484 concentrations of compounds and were incubated at 37°C. On day 7 of culture, the supernatants were
 485 harvested and the amounts of p24 (CA) protein were determined by using Lumipulse G1200. Drug
 486 concentrations that suppressed the production of p24 Gag protein by 50% (EC₅₀; 50% effective
 487 concentration) were determined by comparison with the p24 production level in drug-free control cell
 488 culture. PHA-PBMCs were derived from a single donor in each independent experiment.

489

Compounds

ACAi-028 was purchased from ChemBridge (San Diego, CA, USA). AZT, PF74, and EFV were purchased from Sigma-Aldrich, Ebselen from AdipoGen Life Sciences, and AMD3100 from Selleck Chemicals. Atazanavir (ATV) was kindly provided by Bristol Myers Squibb (New York, NY).

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TN and MA designed and TC, TN, NT, and MA performed all the experiments. NT and MM discussed the data and supported preparation of the MS. MA and HN supervised, managed the project, and acquired the necessary funding. TC, TN, and MA wrote and MM and HN edited the MS. All authors read, commented on, and approved the final manuscript.

We declare that we have no conflicts of interest.

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Table

Table 1: Comparison of anti-HIV-1 activity of ACAi-028 against other inhibitors.

Drugs	EC ₅₀ (μM)			
	Single round inhibition ^a	Multiple round inhibition ^b		
	TZM-bl	MT-4	MT-2	
	HIV-1 _{VSVG-NL4-3}	HIV-1 _{NL4-3}	HIV-1 _{LAI}	HIV-2 _{ROD}
ACAi-028	0.48 ± 0.21	0.12 ± 0.04	0.55 ± 0.04	>10
PF74	0.76 ± 0.55	0.33 ± 0.08	0.37 ± 0.50	>10
Ebselen	1.68 ± 1.45	1.83 ± 0.10	1.73 ± 0.04	n.d.
AZT	0.14 ± 0.13	0.26 ± 0.02	0.23 ± 0.05	n.d.

^a TZM-bl cells (5×10⁴/mL) were subjected to HIV-1_{VSVG-NL4-3} (50 ng/mL of p24) in 96-well plates for 48 h in the presence of the tested compounds. The supernatant was removed and lysis buffer added to the samples. After the samples were shaken for 25 minutes, D-luciferin was added to each well. The luciferase intensity was measured using FluoSTAR Omega.ssay at 48 h. ^b MT-2 cells (10⁴/ml) were exposed to 100 TCID₅₀ of HIV-1_{LAI} or HIV-2_{ROD} and cultured in the presence of various concentrations of each compound, and the EC₅₀ (50% effective concentration) values were determined by the MTT assay. For HIV-1_{NL4-3}, the EC₅₀ values were determined by using MT-4 cells as target cells. MT-4 cells (10⁵/ml) were exposed to 100 TCID₅₀s of HIV-1_{NL4-3}, and the inhibition of p24 Gag protein production by each drug was used as an endpoint. All assays were conducted in duplicate, and the data shown represent mean values (± 1 S.D.) derived from the results of two or three independent experiments.

706

707 **Table 2: Anti-HIV-1 activity of ACAi-028 against clinically isolated multi-drug-resistant HIV-1**
708 **in PBMC and protease-inhibitor-resistant laboratory strain in MT-4 cells.**

Drugs	EC ₅₀ (μM)		
	PBMC		MT-4
	HIV-1 _{ERS104pre}	HIV-1 _{MDR/B}	HIV-1 _{ATV^R5μM}
ACAi-028	0.32 ± 0.07	0.36 ± 0.05	0.64 ± 0.09
PF74	0.29 ± 0.03	0.33 ± 0.02	0.63 ± 0.07
ATV	0.0026 ± 0.0002	0.45 ± 0.01	>1

709 PHA-PBMC (10⁶/ml) or MT-4 cells (10⁵/ml) were exposed to 100 TCID₅₀ of each virus, and the inhibition of p24
710 Gag protein production by each compound was used as an endpoint. All assays were conducted in duplicate, and the
711 data shown represent mean values (± 1 S.D.) derived from the results of two independent experiments. PHA-PBMCs
712 were derived from a single donor in each independent experiment. HIV-1_{ERS104pre} was isolated from treatment-naïve
713 patient and served as wild-type HIV-1. HIV-1_{MDR/B} was originally isolated from AIDS patient who had received 9
714 anti-HIV-1 drugs over the 34 months, and contains following amino acid substitution in the protease-encoding region
715 compared to the consensus type B sequence cited from the Los Alamos database; L10I, K14R, L33I, M36I, M46I,
716 F53I, K55R, I62V, L63P, A71V, G73S, V82A, L90M, and I93L. HIV-1_{ATV^R5μM} was generated previously by long-
717 term selection experiment using HIV-1_{NL4-3} with increasing concentration of ATV (51), and contains following 10
718 amino acid substitution in the protease-encoding region compared to the wild-type HIV-1_{NL4-3}; L23I, E34Q, K43I,
719 M46I, I50L, G51A, L63P, A71V, V82A, and T91A.

720

721 **Table 3: Comparison of ACAi-028 cytotoxicity against other inhibitors in various cell lines.**

Cells	CC ₅₀ (μM)		
	ACAi-028	PF74	Ebselen
Li-7 (hepatocyte)	>100	>100	>20
HLE (hepatocyte)	72.3	>100	>20
HEK293 (kidney)	62.6	>100	>20
MT-4 (lymphocyte)	>100	>100	>20
MT-2 (lymphocyte)	86.6	>100	>20
PBMC	>100	n.d.	n.d.

722 Cells were incubated with the respective compounds for one week before cytotoxicity was quantified using MTT
723 assay.

724

725 **Table 4: Comparison of ACAi-028 binding position and mechanism with other CA inhibitors.**

726

CA	Group	Name	Binding region	C.M.	Stage of inhibition	Ref.
NTD	New	ACAi-028	Q13, S16, T19	↓	Early	
	A	CAP-1	E28, E29, F32, V59, H62	↓	Late	13, 14
		BD-1, BM-1	F32, H62	↓	Late	20
	B	PF74	Q67, K70, T107	↑	Early/Late	15
		(GS-CA1) Lenacapavir	Q67, K70, T107	↑	Early/Late	17, 18
	C	I-XW-053	I37	↓	Early	21
		C1	E98, H120, I124	↓	Late	22, 44
CTD	D	Ebselen	C198, C218	↑	Early/Late	16
	B	PF74	Y169, L172, R173, Q179	↑	Early/Late	15
		(GS-CA1) Lenacapavir	Y169, L172, R173, Q179	↑	Early/Late	17, 18
	C	I-XW-053	R173	↓	Early	21

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C.M., CA Multimerization ↑ Increase, ↓ Decrease

Ref., Reference.

Binding region of ACAi-028 determined using virtual docking simulation (Fig. 1). ACAi-028 is an early stage inhibitor (Fig. 2) that induces reduction of capsid multimerization (Fig. 7). Please refer to the references listed for details of the mechanisms of the other CA inhibitors.

734 Figure legends

735 **Figure 1. Profile of CA, target cavity, and *in silico* docking simulations of ACAi-**

736 **028**

737 (A) 3D structures of full-length CA (PDB accession number 4XFX) shown in tan, and closed-up of

738 ACAi-028-targeted cavity in the CA-NTD shown in light blue are on the left side. The CA comprises

739 a β -hairpin in green, CypA-BL in purple, eleven α -helices: H1-11 in pink, and a 3_{10} -helix in yellow on

740 the right side. (B) The procedure of *in silico* screening to identify as candidate anti-CA inhibitors. (C)

The chemical structure of ACAi-028. **(D)** The docking simulation result of ACAi-028 with the target cavity is shown. Hydrogen bond interactions between molecular surface of CA CA-NTD_{1-146/Δ87-99G} crystal and ACAi-028 are indicated in dotted lines. The carbons of CA and ACAi-028 are shown in tan and white colors, respectively. Nitrogen atoms, oxygen atoms, hydrogen atoms, and bromine atoms are shown in blue, red, white, and brown, respectively. ACAi-028 forms two H-bonds interaction with the side-chains of Gln13 and Thr19 (inter-atomic distances of 1.87 Å and 1.89 Å, respectively) and H-bond interaction with the main-chain of Ser16 (inter-atomic distance of 2.06 Å). Molecular graphics was performed with UCSF Chimera (<https://www.rbvi.ucsf.edu/chimera>). All docking simulations were performed with SeeSAR and FlexX version 10 (BioSolveIT GmbH, Sankt Augustin, Germany).

Figure 2. The early-stage inhibition of ACAi-028 in the HIV-1 life cycle

(A) Fusion assay was conducted between 293T and COS-7 cells in the presence of ACAi-028 or AMD3100. Results of the luciferase intensity are shown as percentage compared to Dimethyl sulfoxide (DMSO) controls. AMD3100 is a fusion inhibitor against CXCR4 co-receptors of X4-tropic HIV-1.

(B) The early-stage inhibition of ACAi-028 against X4-tropic (HIV-1_{LAI} and HIV-1_{NL4-3}), R5-tropic (HIV-1_{Ba-L} and HIV-1_{JR-FL}), VSV-G HIV-1_{NL4-3}, and HIV-2_{ROD} strains were measured using TZM-bl cells. Luciferase activity was measured at 48 h post-infection and is shown as a percentage normalized to DMSO controls.

(C) Time-of-addition assay was conducted by the addition of ACAi-028 and various early-stage inhibitors such as AMD3100, AZT, EFV, PF74, and RAL to TZM-bl cells in 2 h

intervals up to 10 h. Data was shown as percentage every 2 h and normalized to DMSO controls (**D**) Effects of ACAi-028, PF-74, and EFV on RT activity were measured by colorimetric reverse transcriptase assay. OD₄₀₅ values were measured and shown as percentages. All assays were performed in duplicate, and error bars indicate $\pm$ SD from at least two independent experiments. Statistical significance was examined using Student's t-test; *, $P < 0.05$, **, $P < 0.005$.

Figure 3. Effect of ACAi-028 on the latestage of the HIV-1 life cycle

(**A**) Gag-pol proteolytic processing and virus production were examined by the western blotting with anti-Gag antibody in the cell lysate and (**B**) measuring the p24 levels in the supernatant of 293T cells which were transfected with pNL₄₋₃ in the presence of ACAi-028 (10 μ M), PF74 (10 μ M), Ebselen (10 μ M), or DRV (2 μ M). After the virions in the supernatants were purified, Gag-pol proteolytic processing and HIV-1 maturation of the virions were examined by (**C**) the western blotting with anti-Gag on the left side and anti-IN antibody on the right side, and by (**D**) TZM-bl assay at the bottom. All assays were performed in duplicate, and error bars indicate $\pm$ SD from three independent experiments. Statistical significance was examined using Student's t-test; *, $P < 0.05$, **, $P < 0.005$.

Figure 4. Amino acid residues and structures of CA-NTD among HIV and SIV

777 (A) Alignment of CA-NTD (residues from 1 to 45) among laboratory HIV-1 strains, SIV_{CPZ}, HIV-2
778 strains, and SIV_{smn}. (B) Representation of frequencies of CA-NTD (residues from 1 to 50) from 6144
779 sequences of all HIV-1 subtypes (Los Alamos HIV sequence database filtered web alignment) using
780 the WebLogo 3.7.4 application (<http://weblogo.threeplusone.com/create.cgi>). Highlighted in red are
781 the five residues of (Pro1, Gin13, Ser16, Thr19, and Glu45) that constitute the ACAi-028 binding
782 cavity. Above each of these residues is the percentage consensus as determined by Jalview 2.11.1.4
783 program (<http://www.jalview.org>) (52). (C) The target cavity on the surface of CA-NTD_{1-146/Δ87-99G}
784 monomer used in the flexible docking simulation is shown in tan in the left panel. CA-NTD_{HIV-2} (CA-
785 NTD_{ROD}) monomer ([PDB] accession number 2WLV) and corresponding target cavity is shown in
786 cyan in the right panel. The bottom panel shows merged image of the CA-NTD of both strains. (D)
787 The docking simulation result of ACAi-028 with the target cavity in CA-NTD_{ROD} is shown. Hydrogen
788 bond interactions between molecular surface of CA-NTD_{ROD} and ACAi-028 were not observed unlike
789 the docking result between CA-NTD_{HIV-1} and ACAi-028. The carbons of CA and ACAi-028 are shown
790 in cyan and white colors, respectively. Nitrogen atoms, oxygen atoms, hydrogen atoms, and bromine
791 atoms are shown in blue, red, white, and brown, respectively. Docking simulations were performed
792 with SeeSAR and FlexX version 10. Molecular graphics was performed with UCSF Chimera.

793

794 **Figure 5. ACAi-028 interacts directly and non-covalently with CA monomer**

ESI-MS spectra of ACAi-028 (50 μ M) and Ebselen (50 μ M) binding to CA. (A) Black arrows represent CA monomer peaks of the charged ions. Deconvoluted ESI-MS spectrum of CA monomer is shown on the right side. (B) ESI-MS spectra of CA binding to ACAi-028 are shown in red arrows. Deconvoluted ESI-MS spectra of CA monomer or CA monomer binding to ACAi-028 are shown on right side. (C) Under denaturing condition, acetonitrile and trifluoroacetic acid are added to a mixture CA of ACAi-028. Black arrows indicate CA monomers failed to interact with ACAi-028. Deconvoluted ESI-MS spectra is shown on the right side. (D) Under the same condition as (C), black arrows indicate CA monomers and red arrows represent CA monomer binding covalently to one (left) or two (right) ebselen, respectively. Each deconvoluted ESI-MS spectrum is shown on the right side.

Figure 6. Effect of ACAi-028 on CA thermal stability.

(A) DSF was performed using SYBR-Orange dye in the presence of methanol shown in black circle, a different concentration (1, 10, and 50 μ M) of ACAi-028 shown in grey squares, and PF74 (10 μ M) shown in black triangle to examine CA stability. (B) Graphical representation of Tm changes (Δ Tm 50) from (A) are shown for ACAi-028 and PF74. (C) DSF of CA_{S16E} and CA_{T19A} were tested in high concentration of ACAi-028 (10 μ M and 50 μ M shown in light and dark grey squares, respectively). Black circle indicates in methanol as a control. (D) Graphical representation of Δ Tm 50 from (C) are shown for (10 and 50 μ M) ACAi-028. All assays were performed in triplicate, and error bars indicate

±SD from three independent experiments. Statistical significance was examined using Student's t-test (*, $P < 0.05$, **, $P < 0.005$).

Figure 7. Effect of ACAi-028 on CA multimerization.

(A) CA multimerization assay was performed by the addition of a high sodium buffer (the ratio of 150 mM sodium phosphate to 5M sodium phosphate buffer is five to five) in the presence of ACAi-028 (4 and 40 μ M), PF74 (4 μ M), and RAL (40 μ M). Turbidity of the mixtures was measured at OD₃₅₀ over a period of 60 minutes. PF74 and RAL are used as positive or negative controls, respectively. Representative data is shown from three independent experiments. (B) CA_{WT} multimerization was compared to CA_{T19A}, CA_{S16E}, and CA_{M185A} multimerization (The ratio of 150 mM to 5M sodium is five to five). Effects of ACAi-028 (4 and 40 μ M) on (C) CA_{S16E} multimerization in higher sodium concentration (the ratio of 150 mM to 5M sodium is four to six), and (D) CA_{T19A} multimerization (the ratio of 150 mM to 5M sodium is five to five) are shown. Representative data is shown from three independent experiments.

Figure 8. Interaction of ACAi-028 with the binding pockets of CA in the hexameric state.

(A) Left panel shows the structure of a CA hexamer ([PDB] accession number 3H4E). CA dimer extracted from the CA hexamer was used in the flexible docking simulation. Right panel shows the

832 CA hexamer with docking poses of ACAi-028. **(B)** The structure of a CA dimer extracted from a CA
 833 hexamer ([PDB] accession number 5MCX) and the docking result of ACAi-028 to the CA dimer is
 834 shown. ACAi-028 formed two H-bond interactions with the main-chains of Leu43 and Glu45 residues
 835 which are located at the monomer-monomer interface of one CA monomer in the CA dimer extracted
 836 from the CA hexamer. Docking simulations were performed with SeeSAR and FlexX version 10.
 837 Molecular graphics was performed with UCSF Chimera.

838

839 **Figure 9. Profiles of ACAi-028 and CA inhibitors.**

840 ACAi-028 and representative CA inhibitors previously reported are categorized into groups new, A, B,
 841 C, and D. Names, chemical structures, effects of CA multimerization, inhibition stages of the HIV-1
 842 life cycle, putative and representative binding regions of the CA inhibitors are shown.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

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Figure 8

Figure 9