Alkyl Amine Bevirimat Derivatives are Potent and Broadly Active HIV-1 Maturation Inhibitors

Emiko Urano[1], Sherimay D. Ablan[1], Rebecca Mandt[1], Gary T. Pauly[2], Dina M. Sigano[2], Joel P. Schneider[2], David E. Martin[3], Theodore J. Nitz[3], Carl T. Wild[3], and Eric O. Freed#[1]

[1]Virus-Cell Interaction Section, HIV Dynamics and Replication Program, Center for Cancer Research, National Cancer Institute, Frederick, MD 21702-1201

[2]Chemical Synthesis Group, Chemical Biology Laboratory, Center for Cancer Research, National Cancer Institute, Frederick, MD 21702-1201

[3]DFH Pharma, Gaithersburg, MD

#Corresponding author Email: efreed@nih.gov

Running title: Potent HIV-1 maturation inhibitors

ABSTRACT

Concomitant with the release of human immunodeficiency virus type 1 (HIV-1) particles from the infected cell, the viral protease cleaves the Gag polyprotein precursor at a number of sites to trigger virus maturation. We previously reported that a betulinic acid-derived compound, bevirimat (BVM), blocks HIV-1 maturation by disrupting a late step in protease-mediated Gag processing: the cleavage of the capsid-spacer peptide 1 (CA-SP1) intermediate to mature CA. BVM was shown in multiple clinical trials to be safe and effective in reducing viral loads in HIV-1-infected patients. However, naturally occurring polymorphisms in the SP1 region of Gag (e.g., SP1-V7A) led to a variable response in some BVM-treated patients. The reduced susceptibility of SP1-polymorphic HIV-1 to BVM resulted in the discontinuation of its clinical development. To overcome the loss of BVM activity induced by polymorphisms in SP1, we carried out an extensive medicinal chemistry campaign to develop "second-generation" maturation inhibitors. In this study, we focused on alkyl amine derivatives modified at the C-28 position of the BVM scaffold. We identified a set of derivatives that are markedly more potent than BVM against an HIV-1 clade B clone (NL4-3) and show robust antiviral activity against a variant of NL4-3 containing the V7A polymorphism in SP1. One of the most potent of these compounds also strongly inhibited a multi-clade panel of primary HIV-1 isolates. These data demonstrate that C-28 alkyl amine derivatives of BVM can, to a large extent, overcome the loss of susceptibility imposed by polymorphisms in SP1.

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1), the primary causative agent of AIDS, is currently estimated to infect >33 million people worldwide (http://www.healthline.com/health/hiv-aids/facts-statistics-infographic). A number of inhibitors have been developed that suppress HIV-1 replication in infected patients, and currently more than two dozen anti-HIV-1 drugs are approved for clinical use (1). These inhibitors, which are administered in combination (combination antiretroviral therapy, cART), fall into several major classes. Inhibitors of the viral enzymes reverse transcriptase (RT), protease (PR), and integrase (IN) form the backbone of current cART regimens. Inhibitors that target fusion and entry are also available (1).

Although current cART is able to suppress viral loads to below the level of detection in standard commercial assays in the majority of treatment-compliant individuals, available therapies are not curative and thus require lifelong drug adherence. Long-term treatment is associated with a variety of issues related to drug toxicity, unfavorable drug-drug interactions, and patient non-compliance. Multi-drug resistance is likely to limit treatment options in an increasing number of patients over time, particularly in resource-limited settings in which viral load testing is not widely available (2-5). Thus, it is imperative that continued efforts are made to develop novel drugs targeting steps in the viral replication cycle not affected by current therapies. As an added benefit, developing inhibitors against novel targets provides a wealth of basic mechanistic information about fundamental aspects of viral replication.

Maturation of HIV-1 particles, which is triggered by the action of the viral PR, occurs concomitant with virion release from the infected cell (6-8). PR cleaves a number

of sites in the Gag polyprotein precursor, $Pr55^{Gag}$, the major structural protein responsible for the formation of virus particles. PR-mediated Gag cleavage gives rise to the matrix (MA), capsid (CA), nucleocapsid (NC), and p6 proteins and two small spacer peptides, SP1 and SP2, located between CA and NC and between NC and p6, respectively. PR also cleaves the GagPol polyprotein precursor to generate the mature viral enzymes: PR, RT, and IN. Cleavage of the Gag and GagPol polyproteins results in a marked change in virion morphology. In the immature particle, the Gag precursor proteins are arranged radially around the outer edge of the virus particle, whereas in the mature virion the CA proteins assemble into a centrally located, conical core (referred to as the capsid) in which the viral RNA genome and the viral enzymes RT and IN reside. Both $Pr55^{Gag}$ and mature CA assemble into a largely hexameric lattice, though the unit-to-unit spacing of the lattice and the intersubunit contacts differ between the immature and mature lattice (9). The strain of curvature is accommodated in the immature Gag lattice by the presence of gaps, whereas in the mature capsid the inclusion of a total of 12 pentamers in the otherwise hexameric capsid lattice allows the capsid to close off at both ends (10-12). Maturation is critical to particle infectivity (7).

Each processing site within the Gag and GagPol polyprotein precursor is cleaved by PR with distinct kinetics, largely due to the unique primary amino acid sequences at each site (13-19). The consequence of these differential rates of cleavage is that Gag and GagPol processing occurs as a highly ordered cascade of cleavage events. This highly ordered processing is required for proper maturation. Defects in maturation can affect both virus entry (20, 21) and subsequent post-entry events. Even partial disruption of

processing at several sites in Gag leads to severely impaired virus infectivity (22-24), highlighting the utility of Gag processing as a target for antiretrovirals.

We and others previously reported that the first-in-class HIV-1 maturation inhibitor 3-*0*-*(3'*-*3'*-dimethylsuccinyl) betulinic acid (25) (bevirimat or BVM) acts by blocking cleavage at the CA-SP1 junction (26, 27). We selected a panel of resistant mutants by propagating HIV-1 in the presence of BVM; the resistance mutations mapped to the CA-SP1 boundary region (28). Clinical trials showed that BVM was safe and efficacious. However, polymorphisms present in circulating strains of HIV-1 diminished the efficacy of BVM. These polymorphisms cluster in a Gln-Val-Thr (QVT) sequence spanning SP1 residues 6-8. In particular, we observed that in culture, a Val-to-Ala change at SP1 residue 7 (V7A) almost completely abrogated the ability of BVM to block CA-SP1 processing and virus replication (29). Our analysis of the structurally distinct maturation inhibitor PF-46396 (30) demonstrated that this compound retained activity against a derivative of clade B NL4-3 bearing the V7A change (31). This result, together with recent reports describing chemically modified BVM derivatives (32-34), suggested that the insensitivity of SP1 polymorphic HIV-1 to maturation inhibitors could be overcome.

In this study, we synthesized a collection of novel C-28 alkyl amine derivatives of BVM and tested their ability to block CA-SP1 processing and virus replication using WT NL4-3 and an V7A variant of NL4-3 that is resistant to BVM. The activity of these compounds against a multi-clade panel of HIV-1 isolates was also determined. The results demonstrated that several of these derivatives were markedly more potent and

more broadly active than the parental BVM, with low-nM antiviral activity against both WT NL4-3 and the V7A derivative.

Material and Methods

Chemical synthesis. Scaffold **7** compounds (**Table 1**) were synthesized by modification of C3 and C28 of betulin. Oxidation of C28 allowed acylation followed by deprotection at C3 to provide the left-hand side of the molecule. Further homologation following by oxidation and reductive animation at C28 furnished the right-hand side of the molecule resulting in compounds **7a-7t (Table 1). For additional details,** see supplemental material.

Cell culture, plasmids, and transfections. The MT-4 T-cell line was maintained in RPMI-1640 medium supplemented with 10% (vol/vol) fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. HeLa and 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (vol/vol) FBS, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Molecular clones used in this study were WT pNL4-3 (35) and the derivative encoding a Val-to-Ala mutation at SP1 residue 7, referred to as V7A (29). The MT-4 T-cell line was transfected by using the DEAE dextran-mediated procedure; HeLa and 293T cells were transfected with linear polyethylenimine (L-PEI) or Lipofectamine 2000 (Invitrogen) (36, 37).

CA-SP1 accumulation assay. CA-SP1 accumulation assays were performed as described previously (28, 31, 37) with some modification. Briefly, HeLa cells were transfected with WT pNL4-3 or the V7A derivative. At 24 h posttransfection, cells were starved in Met/Cys-free medium for 30 min and metabolically labeled with [^{35}S]Met/Cys-Pro-mix (Perkin Elmer) for 2-3 h. Maturation inhibitors were maintained in the cultures

throughout the transfection and labeling period. Viruses were collected by ultracentrifugation at 75,000× g for 45–60 min. Virus pellets were resuspended in Triton X-100 lysis buffer [300 mM NaCl, 50 mM Tris-HCl (pH 7.5), 0.5% Triton X-100, 10 mM iodoacetamide, and protease inhibitor cocktail tablets (Roche)]. CA and CA-SP1 proteins were separated gels containing between 13.5 and 15% polyacrylamide by SDS-polyacrylamide gel electrophoresis, exposed to a phosphorimager plate (Fuji) and quantified by Quantity One software (Bio-Rad).

Antiviral assays. To produce HIV-1 stocks, 293T cells were transfected with WT pNL4-3 or the V7A derivative and culture supernatants were collected 24 h post transfection. RT-normalized virus supernatants were used to infect MT-4 cells at room temperature for 30 min. Cells were then cultured in the presence of serial dilutions of each compound. At 4 days post-infection, the culture supernatants were subjected to RT assay to monitor viral replication as previously described (37). The 50% inhibitory concentrations (IC_{50}s) were defined as compound concentrations that reduced RT levels to 50% of those measured in the absence of inhibitor (DMSO only controls) using GraphPad Prism 6 software (38). The IC_{50}s in primary peripheral blood mononuclear cells (PBMCs) were determined with a multi-clade panel of HIV-1 isolates by Southern Research Institute (Frederick, MD) as reported previously (38). PBMCs were purified by ficoll-hypaque gradient centrifugation from anonymized, healthy blood donors. Cells were stimulated for three days with phytohemagglutinin and interleukin-2 prior to infection with HIV-1. IC_{50}s were calculated based on reductions in RT activity on day 6 post-infection as described above.

Antimicrobial Agents and Chemotherapy

Cytotoxicity assays. Cytotoxicity was measured with CellTiter-Blue® Cell Viability Assay according to the manufacturer's protocol (Promega). Two cell lines, MT-4 and HeLa, were used in this assay. Cells were incubated with a serial dilution of compounds at 37 ˚C for 4 or 8 days and treated with CellTiter-Blue Reagent. The fluorescent signal was measured at 560 nm Ex /590 nm Em by a plate reader (TECAN, Infinite® M1000 PRO). The 50% cytotoxic concentrations (CC_{50}s) were defined as compound concentrations at which the fluorescent signals were reduced by 50% relative to the no-inhibitor (DMSO only) controls using GraphPad Prism 6 software. Cytotoxicity in PBMCs was determined as described previously (38).

Antimicrobial Agents and Chemotherapy

RESULTS

C-28 BVM analogs inhibit CA-SP1 processing of both WT HIV-1_{NL4-3} and the SP1-V7A derivative. Previous results from this laboratory indicated that compounds containing scaffold **7** possessed some activity against V7A (data not shown). To identify BVM analogs with increased potency and breadth of activity, we synthesized a panel of related C-28 homologated amines (Materials and Methods; Supplemental Information; **Table 1**). We then tested the ability of these derivatives to inhibit CA-SP1 processing of a clade B viral clone, NL4-3, and the BVM-resistant V7A derivative (29). HeLa cells transfected with pNL4-3 or the V7A derivative were left untreated or were treated with 100 nM of each compound **7a-7j** and were metabolically radiolabeled with [^{35}S]Met/Cys. Released virus particles were concentrated by ultracentrifugation and lysates were subjected to SDS-PAGE and fluorography (see Materials and Methods). This approach evaluates the ability of a maturation inhibitor to block CA-SP1 processing in a quantitative manner by measuring the percentage of CA-SP1 accumulation. As indicated in **Fig. 1A**, treatment with BVM or with analogs **7a-7j** led to a marked accumulation (~50-80%) of CA-SP1 for WT NL4-3. In contrast, and consistent with our previous report (29), for samples transfected with the V7A mutant BVM treatment resulted in only ~ 10% accumulation of CA-SP1, which is comparable to the DMSO control (**Fig. 1B**). For the remaining compounds in this set, levels of CA-SP1 accumulation ranged from ~15-55%, with compounds **7h** and **7j**, which incorporate an additional amino grouping into the C-28 side chain, showing the greatest potency in disrupting CA-SP1 processing. Based on these results, we synthesized a new set of compounds (**7k-7t**) and evaluated their potency in the CA-SP1 accumulation assay. Compared to BVM, compounds **7k-7t**

Antimicrobial Agents and Chemotherapy

were considerably more effective against WT NL4-3 (**Fig. 1C**) and showed a high degree of potency against the V7A mutant (**Fig. 1D**) with V7A CA-SP1 accumulation in the 60% range for many of these compounds (**Fig. 1D**).

To refine our analysis of CA-SP1 accumulation, we focused on compounds with the lowest IC_{50} values against V7A, namely **7m**, **7n**, **7r**, and **7s,** and performed a dose-response analysis. Virus-producer cells were treated with increasing concentrations of compound from 0.8 to 100 nM. WT NL4-3 showed a dose-dependent accumulation of CA-SP1 when cells were treated with BVM while SP1-V7A was resistant to all concentrations used (**Fig. 2A and B**). In contrast, compounds **7m**, **7n**, **7r**, and **7s** showed a strong dose-responsive accumulation of CA-SP1 for both WT NL4-3 (**Fig. 2A**) and the V7A mutant (**Fig. 2B**).

C-28 BVM analogs display low-nanomolar antiviral activity against WT NL4-3 and the SP1-V7A derivative. Our previous studies established a close correlation between the inhibition of CA-SP1 processing imposed by BVM and the compound's antiviral activity (28, 29, 31). To determine whether this correlation extends to the BVM analogs being studied here, we used a virus replication assay based on spreading HIV-1 infection in the MT-4 T-cell line to measure the IC_{50} of these compounds. HIV-1-infected MT-4 cells were cultured for four days in the presence of a serial dilution of each compound. Antiviral activity was monitored by reductions in RT activity in culture supernatants. As shown in **Table 2**, the IC_{50}s of the first series of compounds against WT NL4-3 were mainly in the 10-20 nM range. Against V7A, compound **7h** and **7j** had IC_{50}s of 76 and 39 nM, respectively. The significantly improved antiviral activity of **7h** and **7j** against V7A parallels the increased ability of these compounds to inhibit CA-SP1 processing relative

to BVM (**Fig. 1B**). Most of the second series of compounds (**7k-7t**) displayed single-digit nM IC_{50} values against WT and predominantly sub-20 nM IC_{50} values against V7A. Compound **7m**, **7n**, **7r**, and **7s** showed low-nM activity against SP1-V7A (~9, 14, 10, and 8 nM, respectively). As seen with **7s** and **7t**, additional substitution on the second amine grouping is tolerated and will allow for continued exploration of the structure-activity relationship for this series of analogs. We also evaluated the cytotoxicity of these BVM analogs by using the CellTiter-Blue cell viability assay in HeLa and MT-4 cells (see Materials and Methods). As shown in **Table 3**, although many of the highly potent compounds displayed increased cytotoxicity in HeLa and MT-4 cells relative to BVM, the CC_{50} values for all the BVM analogs were considerably higher than the concentrations required for maximal (plateau) antiviral activity.

C-28 BVM analogs are broadly active against multiple clades of HIV-1. Polymorphisms in SP1 (e.g., V7A) reduce susceptibility of HIV-1 to BVM both in *in vitro* assays and *in vivo* in HIV-1-infected patients. To assess the breadth of antiviral activity displayed by our novel panel of BVM analogs, we extended our analyses to a panel of primary HIV-1 isolates that includes representative viruses from HIV-1 subtypes A, B, C, D, E, F, and G. This panel of isolates was selected in part because of their highly diverse SP1 sequences (**Fig. 3**). Compound **7r** was selected for this analysis because of its high level of potency against V7A. Antiviral activity was measured in PBMCs by quantifying the reduction in RT activity in the culture supernatant six days post-infection. BVM displayed low-nM IC_{50}s against only some of these isolates (**Table 4**). In contrast, compound **7r** was active across all of the primary isolates except for the subtype A isolate 92UG031. This virus contains both CA-V230I and SP1-V7A

Antimicrobial Agents and Chemotherapy

polymorphisms (**Fig. 3**). Our preliminary resistance data suggest that the combination of the V230I and V7A polymorphisms confers resistance to BVM analogs (data not shown). However, the frequency of both of these polymorphisms occurring together is very low, fewer than 1% of more than 30,000 viral sequences compiled in the Los Alamos HIV-1 sequence database (http://www.hiv.lanl.gov/content/index). Cytotoxicity was limited in PBMCs, with the CC_{50} for BVM >10,000 nM and no toxicity observed for compound **7r** up to 1000 nM (data not shown). These data clearly demonstrate that, relative to parental BVM, the C-28 alkyl amine derivatives of BVM display a marked improvement in potency and breadth of activity.

Antimicrobial Agents and Chemotherapy

DISCUSSION

In this study, we conducted an extensive medicinal chemistry campaign to develop second-generation maturation inhibitors that not only demonstrate increased potency against WT HIV-1, but also are active against SP1 polymorphic HIV-1 isolates in the low-nM concentration range. The lack of antiviral activity against HIV-1 isolates with polymorphisms near the CA-SP1 cleavage site, particularly in SP1 residues 6-8, was a major reason for the discontinuation of BVM clinical development. Herein, we synthesized a series of BVM analogs with C-28 modifications, with the top candidates potently inhibiting the processing of CA-SP1 and viral replication of a representative polymorphic virus, V7A. These compounds were also active against a multi-clade panel of HIV-1 isolates in PBMCs. While several of the most potent compounds exhibited increased cytotoxicity in laboratory cell lines relative to BVM, cytotoxicity was not observed up to 1000 nM in PBMC.

Recently, several other groups reported the synthesis and antiviral activity of BVM analogs. Most of these analogs have C-28 modifications and demonstrated slightly improved antiviral activity compared to BVM against prototypic clade B HIV-1 (34, 39, 40). Interestingly, some of these BVM analogs reportedly inhibited both viral maturation and viral entry (32, 41). In our study, the antiviral activity correlated well with CA-SP1 accumulation, and infection with VSV-G-pseudotyped virus was potently inhibited by the BVM derivatives in single-cycle assays. In addition, the infectivity of virions bearing the HIV-1 Env glycoprotein was not affected by the inhibitors when they were present at the time of infection (data not shown). Thus, the compounds synthesized here act as maturation inhibitors, with no evidence of an entry-based inhibitory activity. In addition

Antimicrobial Agents and Chemotherapy

to C-28 modifications, it was reported that position C-3 can be derivatized without losing antiviral activity (39). More recently, Dang et al. identified a C-28 BVM derivative that exhibited 20-fold increased activity against virus bearing the V7A polymorphism (32). A series of macrocyclized betulinic acid derivatives was also reported; however, these compounds were not able to overcome the resistance conferred by the V7A polymorphism (40). The most potent compounds in our study demonstrated IC_{50} values against the V7A polymorph that were >50-fold lower than that of parental BVM. Thus, these compounds significantly contribute to the available information concerning the structure-activity relationship of HIV-1 maturation inhibitors.

The mechanism by which C-28 BVM derivatives potently and broadly inhibit HIV-1 replication relative to the parental compound remains to be determined. The modifications may allow the C-28 side chain of the compound to interact more tightly with the putative Gag substrate. Although the binding site for BVM and its analogs has not been structurally characterized, several lines of evidence support the hypothesis that contacts are made between these compounds and two regions of Gag: the CA-SP1 boundary region and the major homology region (MHR) of CA. An early study demonstrated that BVM is specifically incorporated into immature but not mature particles (42). Association of ^{3}H-labeled BVM with immature particles was reduced by resistance-conferring mutations at the CA-SP1 cleavage site (43). Nguyen et al. demonstrated that a photoaffinity BVM analog interacted with residues around the CA-SP1 cleavage site and the CA MHR (44). In a previous study (31), we demonstrated that resistance to a structurally distinct maturation inhibitor, PF-46396, mapped not only to the vicinity of the CA-SP1 cleavage site (where resistance mutations against BVM map)

but also to the MHR. The MHR mutants were strongly compound-dependent, and could revert in culture by acquiring a mutation at residue 8 of SP1 (SP1-T8I). The ability of an SP1 mutation to rescue the replication defect conferred by an MHR substitution suggested functional cross-talk between the MHR and SP1. It is interesting to note that residue 8 of SP1 falls within the so-called 'QVT' motif (SP1 residues 6-8) previously shown to influence BVM activity (29). Poor solubility of BVM and its analogs has hampered structural characterization of BVM binding, although a BVM derivative with improved solubility has been reported and shown by NMR to bind a CA-SP1-NC peptide (45). Adding to the challenging nature of defining the maturation inhibitor-binding site is the fact that the structure of the CA-SP1 boundary region of Gag is poorly defined. Although recent progress has been made in delineating the structure of the CA domain in the immature Gag lattice, the high level of flexibility of SP1 has thus far precluded an atomic-resolution structure of SP1 in the immature particle (9). Binding of maturation inhibitors to Gag may stabilize SP1 (46, 47) thus potentially facilitating structural studies of this region. Mutations in SP1 may also limit the conformation flexibility of this peptide, providing another useful tool for structural studies.

The second-generation HIV-1 maturation inhibitors developed in this study provide insights into the structure-activity relationship of this class of compounds. Ongoing studies are aimed at further optimizing the potency and breadth of activity of these inhibitors.

309 **ACKNOWLEDGEMENTS**

310 We thank members of the Freed laboratory for helpful discussion and critical review of
311 the manuscript and Wei Shau (National Cancer Institute) for HIV-1 sequence analyses.
312 We gratefully acknowledge Southern Research Institute, Frederick, MD for the
313 multiclade analysis shown in Table 4 and Ioannis Kagiampakis (National Cancer
314 Institute) for assistance with graphpad statistical analyses. Work in the Freed laboratory
315 is supported by the Intramural Research Program of the Center for Cancer Research,
316 National Cancer Institute, NIH, and by the Intramural AIDS Targeted Antiviral Program.
317 This research was supported by Public Health Service grant 1R41AI108457-1 to C.T.W.
318 and contract N01-AI-70041. This project was conducted in part by Southern Research
319 Institute using federal funds from the Division of AIDS, National Institute of Allergy and
320 Infectious Diseases, National Institutes of Health under contract HHSN272200700041C
321 entitled "Confirmatory In Vitro Evaluations of HIV Therapeutics".

322

REFERENCES

1. **Kinch MS, Patridge E.** 2014. An analysis of FDA-approved drugs for infectious disease: HIV/AIDS drugs. Drug Discov Today **19:**1510-1513.
2. **Bertagnolio S, Perno CF, Vella S, Pillay D.** 2013. The Impact of HIV Drug Resistance on the Selection of First- and Second-Line ART in Resource-Limited Settings. J Infect Dis **207 Suppl 2:**S45-48.
3. **Hosseinipour MC, Gupta RK, Van Zyl G, Eron JJ, Nachega JB.** 2013. Emergence of HIV drug resistance during first- and second-line antiretroviral therapy in resource-limited settings. J Infect Dis **207 Suppl 2:**S49-56.
4. **Iyidogan P, Anderson KS.** 2014. Current perspectives on HIV-1 antiretroviral drug resistance. Viruses **6:**4095-4139.
5. **Stadeli KM, Richman DD.** 2012. Rates of emergence of HIV drug resistance in resource-limited settings: a systematic review. Antivir Ther **18:**115-123.
6. **Freed EO.** 2015. HIV-1 assembly, release and maturation. Nat Rev Microbiol.
7. **Konvalinka J, Krausslich HG, Muller B.** 2015. Retroviral proteases and their roles in virion maturation. Virology **479-480C:**403-417.
8. **Lee SK, Potempa M, Swanstrom R.** 2012. The choreography of HIV-1 proteolytic processing and virion assembly. J Biol Chem **287:**40867-40874.
9. **Schur FK, Hagen WJ, Rumlova M, Ruml T, Muller B, Krausslich HG, Briggs JA.** 2015. Structure of the immature HIV-1 capsid in intact virus particles at 8.8 A resolution. Nature **517:**505-508.
10. **Briggs JA, Riches JD, Glass B, Bartonova V, Zanetti G, Krausslich HG.** 2009. Structure and assembly of immature HIV. Proc Natl Acad Sci U S A **106:**11090-11095.
11. **Fuller SD, Wilk T, Gowen BE, Krausslich HG, Vogt VM.** 1997. Cryo-electron microscopy reveals ordered domains in the immature HIV-1 particle. Curr Biol **7:**729-738.
12. **Li S, Hill CP, Sundquist WI, Finch JT.** 2000. Image reconstructions of helical assemblies of the HIV-1 CA protein. Nature **407:**409-413.
13. **Erickson-Viitanen S, Manfredi J, Viitanen P, Tribe DE, Tritch R, Hutchison CA, 3rd, Loeb DD, Swanstrom R.** 1989. Cleavage of HIV-1 gag polyprotein synthesized in vitro: sequential cleavage by the viral protease. AIDS Res Hum Retroviruses **5:**577-591.
14. **Krausslich HG, Schneider H, Zybarth G, Carter CA, Wimmer E.** 1988. Processing of in vitro-synthesized gag precursor proteins of human immunodeficiency virus (HIV) type 1 by HIV proteinase generated in Escherichia coli. J Virol **62:**4393-4397.
15. **Mervis RJ, Ahmad N, Lillehoj EP, Raum MG, Salazar FH, Chan HW, Venkatesan S.** 1988. The gag gene products of human immunodeficiency virus type 1: alignment within the gag open reading frame, identification of

posttranslational modifications, and evidence for alternative gag precursors. J Virol **62:**3993-4002.

16. **Pettit SC, Moody MD, Wehbie RS, Kaplan AH, Nantermet PV, Klein CA, Swanstrom R.** 1994. The p2 domain of human immunodeficiency virus type 1 Gag regulates sequential proteolytic processing and is required to produce fully infectious virions. J Virol **68:**8017-8027.
17. **Tritch RJ, Cheng YE, Yin FH, Erickson-Viitanen S.** 1991. Mutagenesis of protease cleavage sites in the human immunodeficiency virus type 1 gag polyprotein. J Virol **65:**922-930.
18. **Vogt VM.** 1996. Proteolytic processing and particle maturation. Curr Top Microbiol Immunol **214:**95-131.
19. **Wiegers K, Rutter G, Kottler H, Tessmer U, Hohenberg H, Krausslich HG.** 1998. Sequential steps in human immunodeficiency virus particle maturation revealed by alterations of individual Gag polyprotein cleavage sites. J Virol **72:**2846-2854.
20. **Murakami T, Ablan S, Freed EO, Tanaka Y.** 2004. Regulation of human immunodeficiency virus type 1 Env-mediated membrane fusion by viral protease activity. J Virol **78:**1026-1031.
21. **Wyma DJ, Jiang J, Shi J, Zhou J, Lineberger JE, Miller MD, Aiken C.** 2004. Coupling of human immunodeficiency virus type 1 fusion to virion maturation: a novel role of the gp41 cytoplasmic tail. J Virol **78:**3429-3435.
22. **Checkley MA, Luttge BG, Soheilian F, Nagashima K, Freed EO.** 2010. The capsid-spacer peptide 1 Gag processing intermediate is a dominant-negative inhibitor of HIV-1 maturation. Virology **400:**137-144.
23. **Lee SK, Harris J, Swanstrom R.** 2009. A strongly transdominant mutation in the human immunodeficiency virus type 1 gag gene defines an Achilles heel in the virus life cycle. J Virol **83:**8536-8543.
24. **Muller B, Anders M, Akiyama H, Welsch S, Glass B, Nikovics K, Clavel F, Tervo HM, Keppler OT, Krausslich HG.** 2009. HIV-1 Gag processing intermediates trans-dominantly interfere with HIV-1 infectivity. J Biol Chem **284:**29692-29703.
25. **Kanamoto T, Kashiwada Y, Kanbara K, Gotoh K, Yoshimori M, Goto T, Sano K, Nakashima H.** 2001. Anti-human immunodeficiency virus activity of YK-FH312 (a betulinic acid derivative), a novel compound blocking viral maturation. Antimicrob Agents Chemother **45:**1225-1230.
26. **Li F, Goila-Gaur R, Salzwedel K, Kilgore NR, Reddick M, Matallana C, Castillo A, Zoumplis D, Martin DE, Orenstein JM, Allaway GP, Freed EO, Wild CT.** 2003. PA-457: a potent HIV inhibitor that disrupts core condensation by targeting a late step in Gag processing. Proc Natl Acad Sci U S A **100:**13555-13560.
27. **Zhou J, Yuan X, Dismuke D, Forshey BM, Lundquist C, Lee KH, Aiken C, Chen CH.** 2004. Small-molecule inhibition of human immunodeficiency virus type 1 replication by specific targeting of the final step of virion maturation. J Virol **78:**922-929.
28. **Adamson CS, Ablan SD, Boeras I, Goila-Gaur R, Soheilian F, Nagashima K, Li F, Salzwedel K, Sakalian M, Wild CT, Freed EO.** 2006. In vitro resistance

to the human immunodeficiency virus type 1 maturation inhibitor PA-457 (Bevirimat). J Virol **80:**10957-10971.

29. **Adamson CS, Sakalian M, Salzwedel K, Freed EO.** 2010. Polymorphisms in Gag spacer peptide 1 confer varying levels of resistance to the HIV- 1 maturation inhibitor bevirimat. Retrovirology **7:**36.

30. **Blair WS, Cao J, Fok-Seang J, Griffin P, Isaacson J, Jackson RL, Murray E, Patick AK, Peng Q, Perros M, Pickford C, Wu H, Butler SL.** 2009. New small-molecule inhibitor class targeting human immunodeficiency virus type 1 virion maturation. Antimicrob Agents Chemother **53:**5080-5087.

31. **Waki K, Durell SR, Soheilian F, Nagashima K, Butler SL, Freed EO.** 2012. Structural and functional insights into the HIV-1 maturation inhibitor binding pocket. PLoS Pathog **8:**e1002997.

32. **Dang Z, Ho P, Zhu L, Qian K, Lee KH, Huang L, Chen CH.** 2013. New betulinic acid derivatives for bevirimat-resistant human immunodeficiency virus type-1. J Med Chem **56:**2029-2037.

33. **Dang Z, Qian K, Ho P, Zhu L, Lee KH, Huang L, Chen CH.** 2012. Synthesis of betulinic acid derivatives as entry inhibitors against HIV-1 and bevirimat-resistant HIV-1 variants. Bioorg Med Chem Lett **22:**5190-5194.

34. **Qian K, Bori ID, Chen CH, Huang L, Lee KH.** 2012. Anti-AIDS agents 90. novel C-28 modified bevirimat analogues as potent HIV maturation inhibitors. J Med Chem **55:**8128-8136.

35. **Adachi A, Koenig S, Gendelman HE, Daugherty D, Gattoni-Celli S, Fauci AS, Martin MA.** 1987. Productive, persistent infection of human colorectal cell lines with human immunodeficiency virus. J Virol **61:**209-213.

36. **Brissault B, Kichler A, Guis C, Leborgne C, Danos O, Cheradame H.** 2003. Synthesis of linear polyethylenimine derivatives for DNA transfection. Bioconjug Chem **14:**581-587.

37. **Waheed AA, Ono A, Freed EO.** 2009. Methods for the study of HIV-1 assembly. Methods Mol Biol **485:**163-184.

38. **Ptak RG, Gentry BG, Hartman TL, Watson KM, Osterling MC, Buckheit RW, Jr., Townsend LB, Drach JC.** 2010. Inhibition of human immunodeficiency virus type 1 by triciribine involves the accessory protein nef. Antimicrob Agents Chemother **54:**1512-1519.

39. **Qian K, Kuo RY, Chen CH, Huang L, Morris-Natschke SL, Lee KH.** 2010. Anti-AIDS agents 81. Design, synthesis, and structure-activity relationship study of betulinic acid and moronic acid derivatives as potent HIV maturation inhibitors. J Med Chem **53:**3133-3141.

40. **Tang J, Jones SA, Jeffery JL, Miranda SR, Galardi CM, Irlbeck DM, Brown KW, McDanal CB, Han N, Gao D, Wu Y, Shen B, Liu C, Xi C, Yang H, Li R, Yu Y, Sun Y, Jin Z, Wang E, Johns BA.** 2014. Synthesis and Biological Evaluation of Macrocyclized Betulin Derivatives as a Novel Class of Anti-HIV-1 Maturation Inhibitors. Open Med Chem J **8:**23-27.

41. **Huang L, Ho P, Lee KH, Chen CH.** 2006. Synthesis and anti-HIV activity of bi-functional betulinic acid derivatives. Bioorg Med Chem **14:**2279-2289.

42. **Zhou J, Huang L, Hachey DL, Chen CH, Aiken C.** 2005. Inhibition of HIV-1 maturation via drug association with the viral Gag protein in immature HIV-1 particles. J Biol Chem **280:**42149-42155.
43. **Zhou J, Chen CH, Aiken C.** 2006. Human immunodeficiency virus type 1 resistance to the small molecule maturation inhibitor 3-O-(3',3'-dimethylsuccinyl)-betulinic acid is conferred by a variety of single amino acid substitutions at the CA-SP1 cleavage site in Gag. J Virol **80:**12095-12101.
44. **Nguyen AT, Feasley CL, Jackson KW, Nitz TJ, Salzwedel K, Air GM, Sakalian M.** 2011. The prototype HIV-1 maturation inhibitor, bevirimat, binds to the CA-SP1 cleavage site in immature Gag particles. Retrovirology **8:**101.
45. **Coric P, Turcaud S, Souquet F, Briant L, Gay B, Royer J, Chazal N, Bouaziz S.** 2013. Synthesis and biological evaluation of a new derivative of bevirimat that targets the Gag CA-SP1 cleavage site. Eur J Med Chem **62:**453-465.
46. **Keller PW, Adamson CS, Heymann JB, Freed EO, Steven AC.** 2011. HIV-1 maturation inhibitor bevirimat stabilizes the immature Gag lattice. J Virol **85:**1420-1428.
47. **Keller PW, Huang RK, England MR, Waki K, Cheng N, Heymann JB, Craven RC, Freed EO, Steven AC.** 2013. A two-pronged structural analysis of retroviral maturation indicates that core formation proceeds by a disassembly-reassembly pathway rather than a displacive transition. J Virol **87:**13655-13664.

Antimicrobial Agents and Chemotherapy

FIGURE LEGENDS

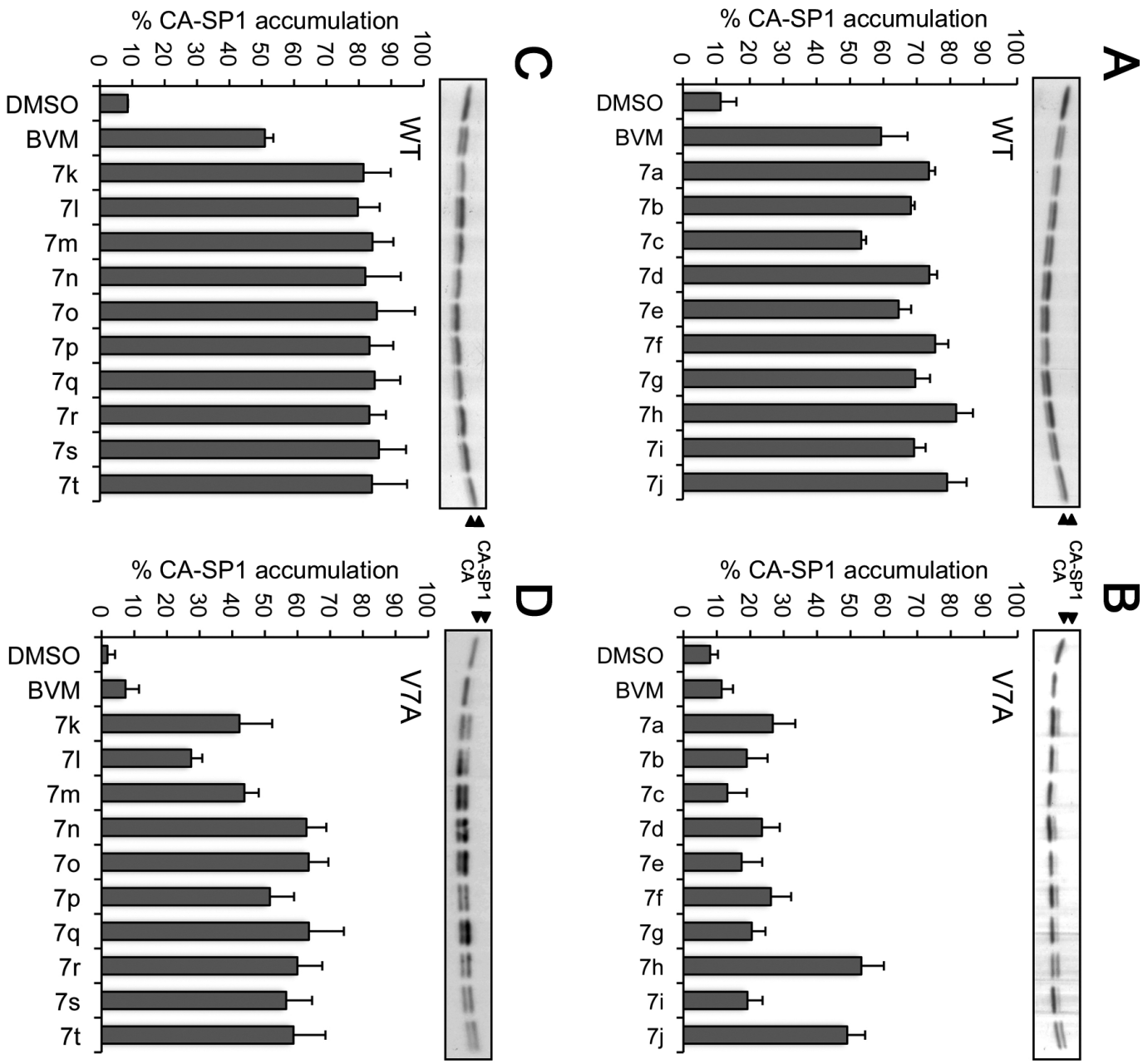
Fig. 1. C-28 alkyl amine BVM derivatives potently disrupt CA-SP1 processing. HeLa cells were transfected with WT pNL4-3 (**A and C**) or the pNL4-3 derivative encoding the V7A polymorph (**B and D**). Cells were treated with 100 nM compounds **7a-7j** (**A and B**) or **7k-7t** (**C and D**) and were metabolically labeled with [^{35}S]Met/Cys in the presence of the compounds. Released virions were collected by ultracentrifugation and virion-associated CA and CA-SP1 were analyzed by SDS-PAGE and quantified by phosphorimager analysis. A representative gel image is shown on the top and quantification of the %CA-SP1 relative to total CA + CA-SP1 is presented in the graphs. Error bars indicate standard deviations from three independent experiments.

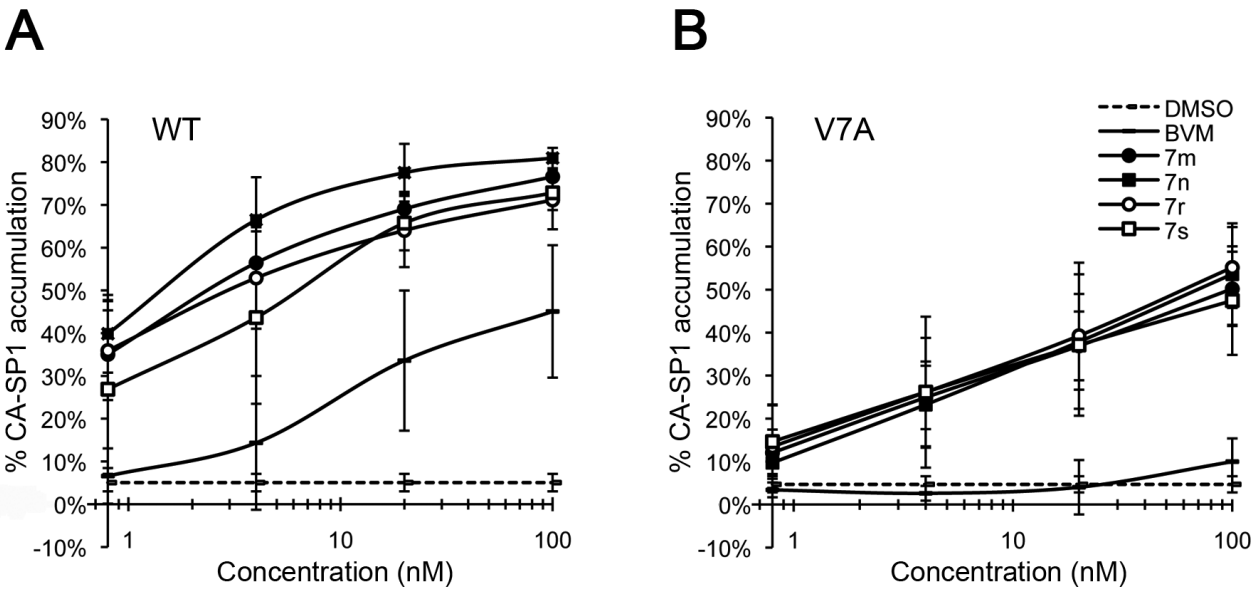
Fig. 2. Dose-dependent inhibition of CA-SP1 processing by the top compounds. HeLa cells were transfected with WT pNL4-3 (**A**) or the pNL4-3 derivative encoding the V7A polymorph (**B**). Cells were treated with a range of concentrations (0.8, 4, 20, and 100 nM) of compounds **7m**, **n**, **r**, or **s** and were metabolically labeled with [^{35}S]Met/Cys in the presence of the compounds. Released virions were collected by ultracentrifugation and virion-associated CA and CA-SP1 were analyzed by SDS-PAGE and quantified by phosphorimager analysis. Quantification of the %CA-SP1 relative to total CA + CA-SP1 is presented in the graphs. Error bars indicate standard deviations from at least three independent experiments.

Fig. 3. Amino acid sequences of the CA-SP1 boundary region from the multi-clade panel of HIV-1 isolates tested in Table 4. Asterisks indicate amino acid sequence identity with

WT NL4-3 (top line); gaps are indicated by hyphens. The QVT motif spanning SP1 residues 6-8 is highlighted in bold.

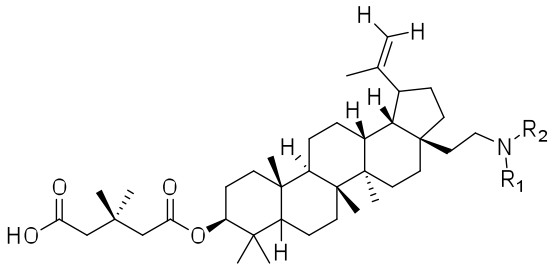
Antimicrobial Agents and Chemotherapy


A
% CA-SP1 accumulation
0 10 20 30 40 50 60 70 80 90 100
DMSO
BVM
7a
7b
7c
7d
7e
7f
7g
7h
7i
7j
WT
B
CA-SP1
CA
% CA-SP1 accumulation
DMSO
BVM
7a
7b
7c
7d
7e
7f
7g
7h
7i
7j
V7A
C
% CA-SP1 accumulation
DMSO
BVM
7k
7l
7m
7n
7o
7p
7q
7r
7s
7t
WT
D
CA-SP1
CA
% CA-SP1 accumulation
DMSO
BVM
7k
7l
7m
7n
7o
7p
7q
7r
7s
7t
V7A

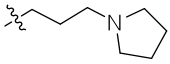
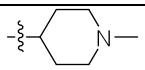


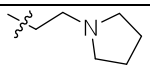
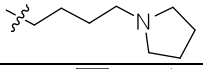
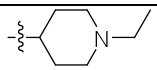
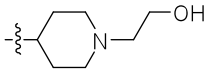
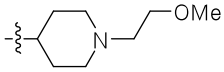
		CA	SP1
NL4-3 WT	Subtype	HKARVL	AEAMS**QVT**N--PATIM
92UG031	A	****I*******	**AQ**H**T-N**
92BR030	B	**********	**M***NA****
93IN101	C	**********	**AN**S**--**L
99UGA07412MI	D	**********	**S**T*NT*-**
CMU02	E	**********	**AQ**H**A-N**
93BR029	F	**********	********SG***
JV1083	G	**********	**AS**GAAA*A**

Table 1. Analogs Prepared and Characterized for This Study



Scaffold 7

Compound #	R_1	R_2	Molecular Formula	Molecular Weight
7a	H	$-CH_2CH_2OH$	$C_{40}H_{67}NO_5$	641.96
7b	$-CH_2CH_2OH$	$-CH_2CH_2OH$	$C_{42}H_{71}NO_6$	686.02
7c	$-CH_2CH_2OCH_2CH_2-$		$C_{42}H_{69}NO_5$	668.02
7d	H	$-CH_2CH_2OCH_3$	$C_{41}H_{69}NO_5$	656.00
7e	$-CH_2CH_2OCH_3$	$-CH_2CH_2OCH_3$	$C_{44}H_{75}NO_6$	714.07
7f	H	$-C(CH_3)_2CH_2OH$	$C_{42}H_{71}NO_5$	670.02
7g	$-CH_2CH_2N(CH_3)CH_2CH_2-$		$C_{43}H_{72}N_2O_4$	681.04
7h	H		$C_{45}H_{76}N_2O_4$	709.09
7i	H	-cyclopropyl	$C_{41}H_{67}NO_4$	637.98
7j	H		$C_{44}H_{74}N_2O_4$	695.07
7k	H	$-CH_2CH_3$	$C_{40}H_{67}NO_4$	625.96
7l	$-CH_2CH_3$	$-CH_2CH_3$	$C_{42}H_{71}NO_4$	654.02
7m	H	$-CH_2CH_2N(CH_3)_2$	$C_{42}H_{72}N_2O_4$	669.03
7n	H	$-CH_2CH_2CH_2N(CH_3)_2$	$C_{43}H_{74}N_2O_4$	683.06
7o	H	$-CH_2CH_2CH_2CH_2N(CH_3)_2$	$C_{44}H_{76}N_2O_4$	697.09

7p	H		$C_{44}H_{74}N_2O_4$	695.07
7q	H		$C_{46}H_{78}N_2O_4$	723.12
7r	H		$C_{45}H_{76}N_2O_4$	709.10
7s	H		$C_{45}H_{76}N_2O_5$	725.10
7t	H		$C_{46}H_{78}N_2O_5$	739.12

Antimicrobial Agents and Chemotherapy

Table 2. Antiviral activity of C-28 alkyl amine derivatives against WT and V7A in MT-4 cells[α]

Compound	IC_{50} (nM)	
	WT	V7A
BVM	136 ± 30	>500
7a	33 ± 26	>500
7b	19 ± 6	>500
7c	98 ± 44	>500
7d	16 ± 7	>500
7e	49 ± 32	>500
7f	9 ± 4	>500
7g	32 ± 16	>500
7h	27 ± 3	76 ± 15
7i	22 ± 14	>500
7j	16 ± 0.4	39 ± 10
7k	3 ± 1	145 ± 29
7l	14 ± 7	201 ± 44
7m	3 ± 0.3	9 ± 2
7n	7 ± 3	14 ± 4
7o	9 ± 3	22 ± 5
7p	3 ± 0.4	26 ± 6.4
7q	5 ± 1	22 ± 7
7r	5 ± 3	10 ± 3
7s	21 ± 19	8 ± 2
7t	2 ± 0.5	19 ± 6

[α]The IC_{50} values represent the mean ± SEM from four independent experiments.

Table 3. **Cytotoxicity of C-28 alkyl amine derivatives in MT-4 and HeLa cells**[α]

Compound	CC_{50} (nM)	
	MT-4	HeLa
BVM	>1000	>1000
7a	>1000	>1000
7b	>1000	>1000
7c	>1000	>1000
7d	>1000	>1000
7e	>1000	>1000
7f	>1000	>1000
7g	>1000	>1000
7h	911 ± 35	>1000
7i	>1000	>1000
7j	>1000	>1000
7k	>1000	>1000
7l	>1000	927 ± 232
7m	425 ± 25	>1000
7n	210 ± 17	381 ± 101
7o	201 ± 21	305 ± 13
7p	377 ± 54	614 ± 33
7q	381 ± 48	291 ± 40
7r	195 ± 13	877 ± 287
7s	366 ± 44	>1000
7t	406 ± 48	551 ± 83

[α]The CC_{50} values represent the mean ± SEM from three independent experiments.

Table 4. **Antiviral activity of C-28 alkyl amine derivatives against a multi-clade panel of HIV-1 isolates in PBMCs**[α]

		IC_{50} (nM)		
	subtype	Compound 7r	BVM	AZT
92UG031	A	≥1000	2829	11
92BR030	B	199	5235	12
93IN101	C	67	2651	19
99UGA07412MI	D	15	15	6
CMU02	E	96	3327	8
93BR029	F	13	7	3
JV1083	G	221	4188	13

[α]Representative data from two independent experiments.