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RESEARCH

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# C-28 linker length modulates the activity of second-generation HIV-1 maturation inhibitors

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## Abstract

Maturation inhibitors (MIs) block HIV-1 maturation by preventing the cleavage of the capsid protein and spacer peptide 1 (CA-SP1). Bevirimat (BVM), a first-in-class MI, displayed sub-optimal efficacy in clinical trials due to presence of SP1:V7A polymorphism in the Gag protein.This polymorphism is inherently present in HIV-1 subtype C and conferred resistance to BVM. Second generation BVM analogs with modifications at C-28 position gained potent activity against HIV-1 subtype C. In this study, we have evaluated the efficacy of nine second-generation MIs (BVM analogs) with varying length of C28 carbon linker against HIV-1 subtype B and C. Increasing the length of carbon linker decreased their activity against both subtypes. These MIs were also active against integrase inhibitor-resistant viruses and protease inhibitor-resistant viruses. Our data has provided vital information that in addition to the nature of the functional group at C28 position of the MI, the length of linker contributes significantly to its activity. The shorter the length, the better the activity of MIs. These results will further pave way for design of novel and potent MIs.

**Keywords** Maturation inhibitors, C-28 Linker length, HIV-1 Subtype B and C, HIV-1 maturation

## Background

Multiple stages of the HIV-1 life cycle are targeted by highly active antiretroviral treatment (HAART). Maturation inhibitors (MIs) target a late step in the viral life cycle by blocking the cleavage of p25 into the p24 capsid protein and spacer peptide 1 (SP1) and prevent the production of infectious virions [1, 2]. MI action may inhibit unfolding of the HIV-1 Gag six-helix bundle and stabilize the immature HIV-1 Gag lattice resulting in production of immature and non-infectious virus [3].

Unlike parental BVM, its analogs modified at C-3, C-28, or C-30 position showed increased potency against a variety of HIV-1 strains, including HIV-1 subtype C [4–8].

HIV-1 subtype C virus infections are predominant in Eastern and Southern Africa and South Asian countries including India, and China. Globally, HIV-1C contributes to nearly 50% of the total HIV-1 infections [9]. The first in class maturation inhibitor, Bevirimat failed in clinical trials due to presence of polymorphism V7A in 'QVT' motif [10, 11]. HIV-1C virus inherently contains naturally occurring polymorphisms -V7A and T8N, in the SP1 region. Hence, it is imperative to screen MIs activity against highly polymorphic HIV-1C viruses.

The activity of MIs against drug- resistant viruses has been previously reported [12]. Challenging MIs with resistant viruses against FDA-approved ARVs (Antiretroviral drugs) has confirmed different mechanisms of

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action of MIs and the possibility of using them in combination therapy.

In this study, we have tested the activity of nine novel second-generation C-28 alkyl amine derivatives of BVM with varying length of carbon linker. We have studied four 1C linker compounds (CV-10237, CV-10238, CV-10239, CV-10240), one 2C linker compound (CV-10236), and four 3C linker compounds (CV-10241, CV-10242, CV-10243 and CV-10244) against multiple HIV-1 subtype B and C strains and protease-inhibitor and integrase inhibitor-resistant viruses.

## Methods

### Preparation of compounds

Second-generation Maturation inhibitors (BVM analogs) were synthesized by chemical modifications at C-28 positions of Bevirimat (BVM) by DFH Pharma, USA. All the compounds were clean and consistent and showed 99% purity by LC/MS. Compounds were dissolved in dimethyl sulfoxide (DMSO) and stored in the dark in − 80 °C freezer.

### Cell culture, plasmids, and transfections

HEK-293 T and HUT-R5 T-cells were kind gifts from Dr. Eric O. Freed, National Cancer Institute, NIH, USA. TZM-bl cells (catalog number: 8129) were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH. HEK-293 T and TZM-bl cells were cultured in complete Dulbecco's modified Eagle's medium (DMEM). HUT-R5 T-cells were maintained in (RPMI)-1640 (Roswell Park Memorial Institute) medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin.

HIV-1 subtype B clone NL4-3 (a kind gift from Dr Eric O. Freed, National Cancer Institute, NIH, USA; GenBank accession: AF324493.2), HIV-1 subtype C clone; K3016 (South African origin, a kind gift from Dr. Christina Ochsenbauer, University of Alabama, USA; GeneBank accession: KC156129), Protease inhibitor-resistant and Integrase inhibitor-resistant clones from the National Institutes of Health AIDS Reagent Program(NIH ARP 11800 and NIH ARP 11847 respectively) [13] were used in this study.

HEK-293 T cells were transfected with 3 µg HIV-1 DNAs (HIV-1 subtype B clone NL4-3, HIV-1 subtype C clone K3016 WT and protease-resistant and integrase resistant mutant clone, NL4-3 11,800 and NL4-3 11,847 respectively) using Lipofectamine 2000 (Invitrogen catalog number: 11668-019).

### CA-SP1 accumulation assay

HEK-293 T cells grown in six-well plates to about 80% confluency were transfected with HIV-1 DNAs (3 µg). The culture medium was replaced with fresh DMEM after 24 h post-transfection and incubated for another 2 h. MIs were maintained in the culture throughout transfection. The viral supernatants were filtered using a 0.45 µm pore size filter disc to remove residual cellular contaminants. The virus was pelleted by ultracentrifugation at 210,100 × g for 1 h at 4 °C using a SW41Ti rotor (Beckman Coulter, USA). To measure the accumulation of CA-SP1, viral lysates were subjected to SDS–polyacrylamide gel electrophoresis (15% gel); proteins were transferred to polyvinylidene difluoride membranes and reacted with HIV-IgG (NIH AIDS Reagent Program; catalog no. 3957) followed by incubation with HRP-conjugated anti-human secondary antibodies (GE Healthcare, UK). The proteins were visualized by enhanced chemiluminescence (BioRad, USA), and the bands were quantified using ImageJ software (http://imagej.nih.gov/ij/).

### Cytotoxicity assay

Cytotoxicity assays were performed using the CellTitre-Blue Cell Viability Assay kit (Promega, USA) as per the manufacturer's recommendations. HEK-293 T and HUT-R5 T-cells were maintained in the presence of serial dilutions of MIs for 48 h and treated with CellTitre-Blue reagent for 4 h at 37 °C. The fluorescent signals were recorded at 530/25 excitation and 590/35 emission using a BioTek microplate reader. The 50% cytotoxicity concentrations ($CC_{50}$s) were determined as the concentrations of MI that reduced the fluorescent signals to 50% relative to DMSO-only controls.

### Antiviral assays

$1.6 \times 10^6$ HUT-R5 T-cells were infected with p24-normalized HIV-1 subtype C K3016 virus (50 ng p24 equivalent) stocks at 37 °C for 2 h. $2 \times 10^4$ virus infected cells were then cultured in 96 well plate in the presence of serial dilutions (500 nM,100 nM,20 nM,4 nm and 0.8 nM) of each compound. The virus supernatants were collected after 8 days, and the concentration of HIV-1 p24 was measured in the virus supernatants. Compared to DMSO-only controls, the doses of MI that decreased HIV-1 p24 concentrations to 50% were determined to be the 50% inhibitory concentrations ($IC_{50}$).

$IC_{50}$ analyses using single-cycle infectivity assays were also performed. HEK-293 T cells were transfected with K3016 and cultured in the presence of serial dilution of MIs. Virus-containing supernatants were harvested, normalized for HIV-1p24 concentration, and used to infect TZM-bl cells. Luciferase activity was measured two days post-infection, and infectivity was calculated. The 50% inhibitory concentrations ($IC_{50}$) were calculated as the concentrations of MI that reduced infectivity to

50% relative to DMSO-only control using the GraphPad Prism V5.0.

### Viral infectivity assay

HEK-293 T cells were transfected with HIV-1 DNAs (HIV-1 subtype B clone NL4-3, HIV-1 subtype C clone K3016 WT, and mutants NL4-3 11,800 and NL4-3 11,847. DMSO or maturation inhibitors (MIs) were maintained in the culture throughout transfection. 24 h post-transfection, culture supernatant was collected and filtered using a 0.45 µm pore size filter disc to remove residual cellular contaminants. The virus stocks were normalized for p24 antigen using an XpressBio ELISA kit (Catalog number: XB-1000). 5 ng HIV-1 p24 equivalent virus was used to infect TZM-bl cells ($5\times10^4$/well) in the presence of 20 µg DEAE-dextran per ml in 24 wells plate. The luciferase activity in the cell lysates was measured 48 h post-infection using the Steady-Glo luciferase assay kit (Promega catalog number: E2510).

### Statistics

All statistical analysis was performed using GraphPad Prism V5.0. One-way ANOVA was done by implying Tukey's test. $p$ values below 0.05, 0.01,0.001 and 0.0001 were denoted as *, **, *** and ****, respectively. Non-significant results were denoted as ns.

## Results

### Novel BVM analogs inhibited HIV-1 subtype B and C viruses

Nine novel BVM analogs (CV-10236, CV-10237, CV-10238, CV-10239, CV-10240, CV-10241, CV-10242, CV-10243 and CV-10244) were synthesized by modifying the C-28 position of BVM with different alkyl amine groups varying in their carbon linker length by DFH Pharma, Gaithersburg, Maryland, USA (Fig. 1). CV-10237, CV-10236, and CV-10241 vary only in the number of carbons (1, 2, or 3 linker carbons) between the triterpene core and the first nitrogen atom of the side chain. CV-10238 and CV-10242 have similar side chains with 1C linker and 3C linker, respectively. Similarly, CV-10239 and CV-10243 differ in their linker lengths-1C linker and 3C linker, respectively. Also, CV-10240 and CV-10244 are 1C linker and 3C linker compounds, respectively. The activity of 2C linker compounds CV8611, CV8612 and CV8613 have been reported by us previously [14].

We tested these maturation inhibitors against HIV-1 subtype B (NL4-3) and C (K3016) viruses. HEK-293 T cells were transfected with plasmid DNAs encoding HIV-1 subtype B or C virus in the absence or presence of 100 nM concentration of the BVM analogs as described in methods. This concentration was chosen as it was the least amount of compound, which gave maximal activity without any toxicity. The virus lysates were analyzed for

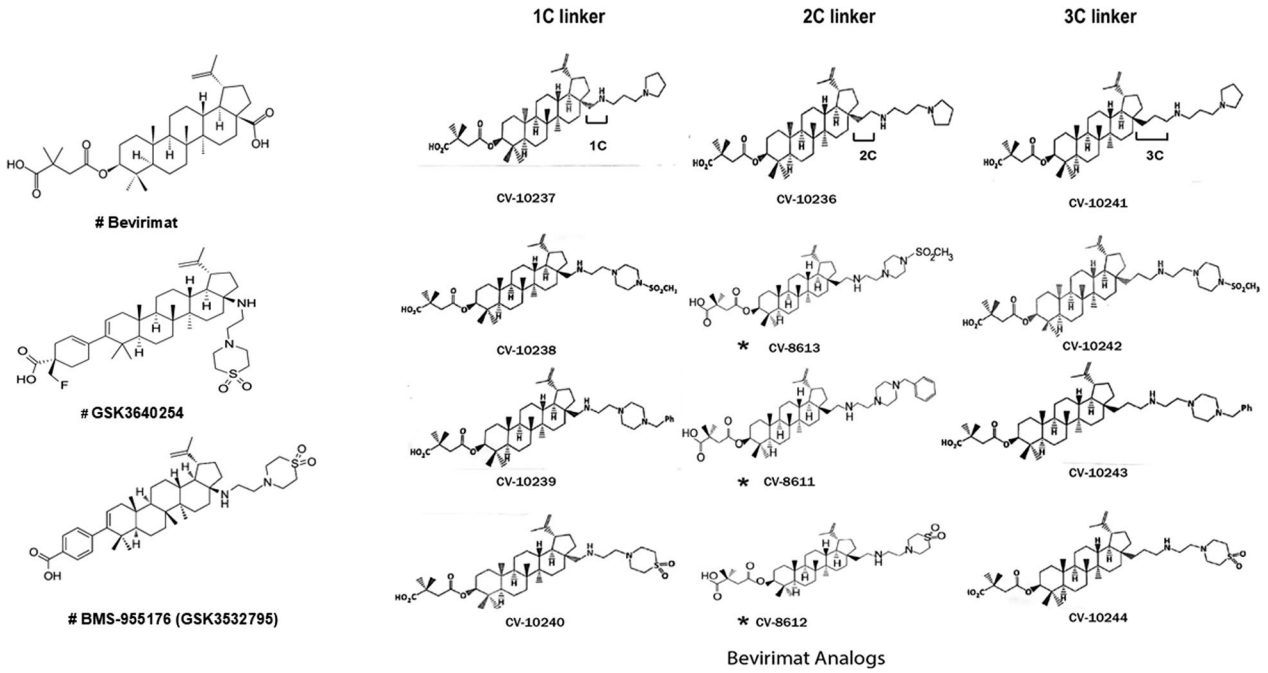


**Fig. 1** Bevirimat Analogs and their carbon linker length derivatives.* Indicates compounds already studied (14). #indicates clinically studied HIV maturation inhibitors [15–17]

accumulation of the CA-SP1 intermediate (p25). There was an increase in the CA-SP1 intermediate in the presence of the compounds, suggesting that the BVM analogs inhibited HIV-1 subtype B Gag processing. We observed 70–75% CA-SP1 accumulation in the presence of 100 nM of CV-10237, CV-10238, CV-10239, and CV-10240, indicating that these BVM analogs prevented the processing of HIV-1 subtype B Gag. CV-10242 showed 50–55% CA-SP1 accumulation, whereas CV-10236 and CV-10241 displayed 25–30% CA-SP1 accumulation at 100 nM concentration. BVM analogs, CV-10243 and CV-10244, were ineffective against HIV-1 subtype B as they showed less than 1% CA-SP1 accumulation (Fig. 2a).

Hence, compounds with 1C linker displayed 70–75% CA-SP1 accumulation, where as 2C and 3C linker compounds exhibited reduced CA-SP1 accumulation (between 1 and 50%).

A TZM-bl cell-based single-cycle infectivity assay was performed to measure the infectivity of the viruses produced in the presence of the compounds [18]. In the presence of 100 nM of 1C linker compounds, there was a 95% reduction in the infectivity of the HIV-1 subtype B virus. In contrast, the infectivity of virus in the presence of 2C and 3C linker compounds was reduced to 40% only. These results confirmed that second-generation BVM analogs with a single linker between the parental compound and the functional groups were more potent in inhibiting HIV-1 subtype B as compared to analogs containing longer linkers.

We next tested the activity of these BVM analogs against the HIV-1 subtype C clone (K3016). We observed 50–60% accumulation of CA-SP1 intermediate in the presence of 100 nM of 1C linker compounds (CV-10237, CV-10238, CV-10239, CV-10240), 30–40% with 2C linker compound (CV-10236), and 5–47% with 3C linker derivatives (CV-10243, CV-10244, CV-10242 and CV-10241) (Fig. 3a).

Viruses produced in the presence of 1C linker compounds had reduced infectivity (60–70%), as compared to up to 30% reduction observed in the presence of 2C and 3C linker BVM analogs (Fig. 3b). These results put together established the broad activity of BVM analogs and confirmed our previous findings that the compounds with single carbon linker were the most potent.

The antiviral activity of MI's against the K3016 virus was calculated by measuring $IC_{50}$ as described in the methods. $IC_{50}$ values of MI's correlated with p25 accumulation and infectivity assay. 1C linker compounds showed lower $IC_{50}$ values compared to 2C and 3C linker compounds (Table 1).

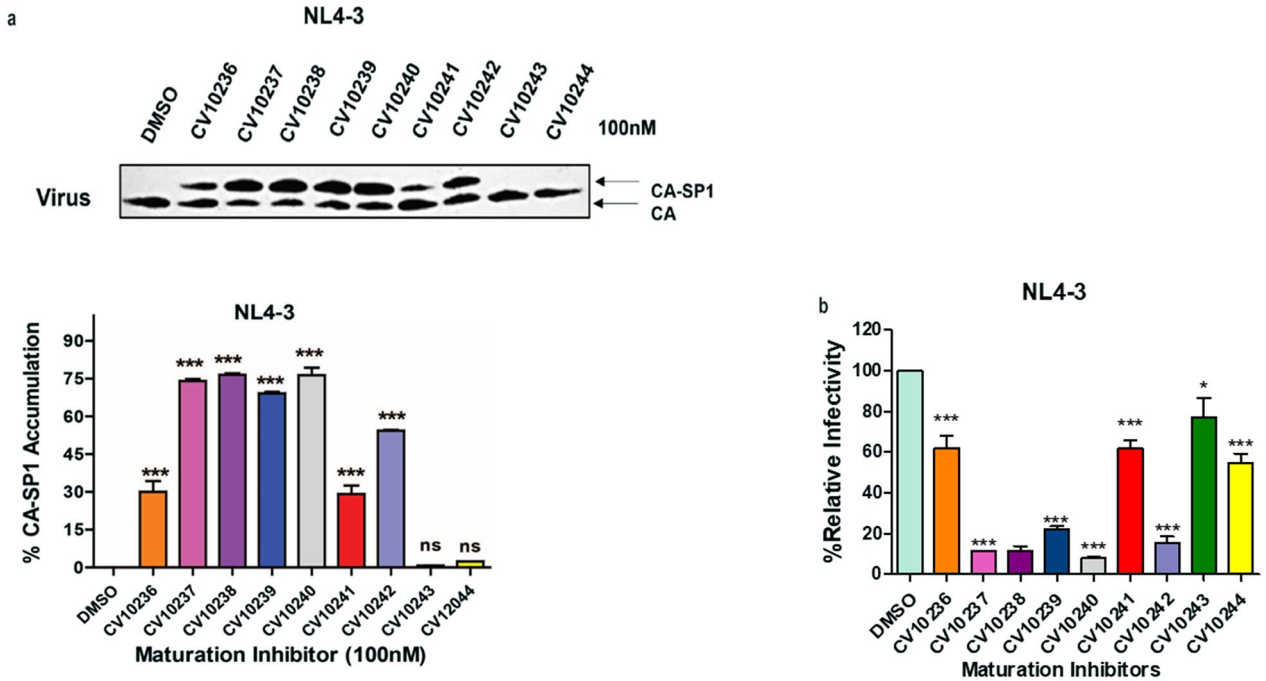


**Fig. 2** Bevirimat analogs are active against HIV-1 subtype B NL4-3 virus. **a** CA-SP1 accumulation assay. HEK293T cells were transfected with HIV-1 subtype B clone NL4-3 treated with 100 nM Bevirimat analogs or with DMSO only. The virion-associated CA and CA-SP1 were detected by western blotting. Quantification of % CA-SP1 relative to total CA-SP1 is presented in the graphs. **b** Infectivity assay of the viruses produced from MI-treated HEK293T cells. Quantitative data for levels of infectivity relative to the DMSO control-treated sample is shown ($n = 3$)

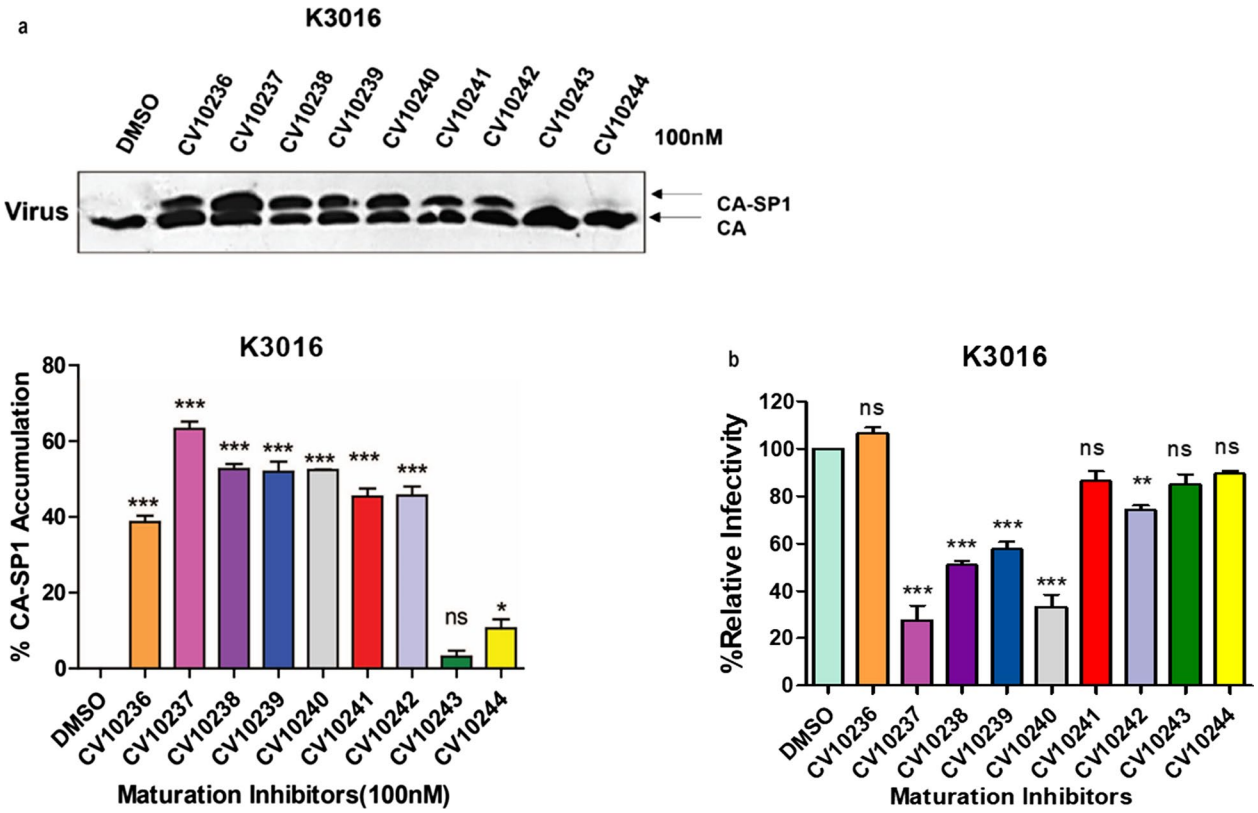


**Fig. 3** Bevirimat analogs are active against the HIV-1 subtype C K3016 virus. **a** CA-SP1 accumulation assay. HEK293T cells were transfected with HIV-1 subtype C K3016 virus. Cells were treated with 100 nM Bevirimat analogs or with DMSO only. The virion-associated CA and CA-SP1 were detected by western blotting. Quantification of % CA-SP1 relative to total CA-SP1 is presented in the graphs. **b** Infectivity assay of the viruses produced from MI-treated HEK293T cells. Quantitative data for levels of infectivity relative to the DMSO control-treated sample is shown ($n = 3$)

**Table 1** Antiviral activity of maturation inhibitors against HIV-1 subtypes C K3016 and B NL4-3 wild type and resistant viruses

| Compounds | $IC_{50}$ (nM) K3016 | $IC_{50}$ (nM) NL4-3 | $IC_{50}$ (nM) NL4-3 11,847 | $IC_{50}$ (nM) NL4-3 11,800 | $CC_{50}$ (μM) (HEK-293Tcells) | $CC_{50}$ (μM) (HUT-R5 T cells) |
|---|---|---|---|---|---|---|
| CV10237 | 2.59 ± 0.26 | 27.07 ± 2.03 | 19.61 ± 0.23 | 17.04 ± 1.13 | 11.80 ± 0.86 | 5.18 ± 0.41 |
| CV10238 | 3.03 ± 0.15 | 31.46 ± 2.77 | 22.78 ± 3.18 | 19.44 ± 0.34 | 17.48 ± 0.44 | 5.60 ± 0.23 |
| CV10239 | 3.28 ± 0.12 | 28.03 ± 0.12 | 30.12 ± 1.08 | 19.64 ± 5.50 | 19.58 ± 2.80 | 5.12 ± 0.17 |
| CV10240 | 3.21 ± 0.08 | 25.57 ± 2.23 | 19.45 ± 0.05 | 24.05 ± 2.31 | 17.49 ± 1.22 | 5.40 ± 0.20 |
| CV10241 | 13.84 ± 0.21 | 176.5 ± 26.44 | 150.8 ± 38.6 | 116.5 ± 10.10 | 12.59 ± 0.62 | 6.33 ± 0.13 |

$IC_{50}$ values represent the mean ± standard deviation of three independent experiments. $CC_{50}$ values represent the mean ± standard deviation of three independent experiments

### BVM analogs retained activity against drug-resistant viruses

The efficacy of the BVM analogs (CV-10237, CV-10238, CV-10239, CV-10240, and CV-10241) on the HIV-1 subtype B WT and the protease-inhibitor (NL4-3 11,800) and integrase inhibitor-resistant viruses (NL4-3 11,847) were analyzed by performing antiviral assay as described in methods. All BVM analogs retained activity against drug-resistant viruses similar to the HIV-1 subtype B NL4-3 wild-type virus. CV-10237 (1C linker compound) displayed lower $IC_{50}$ values as compared to CV-10241 (3C linker compound) (Table 1). These results further validated that BVM analogs containing a single carbon linker were more active than analogs with a longer linker.

$IC_{50}$ values represent the mean ± standard deviation of three independent experiments. $CC_{50}$ values represent

the mean ± standard deviation of three independent experiments.

## Discussion

In this study, we have characterized novel second-generation maturation inhibitors with varying C-28 linker carbon lengths. Maturation inhibitors containing a single carbon linker -CV-10237, CV-10238, CV-10239, and CV-10240 were more active against HIV-1 B and C virus. Compounds with 2C and 3C linkers showed reduced activity against both the subtypes (Figs. 2 and 3). The most potent compound, CV-10237 (1C linker carbon length), exhibited an $IC_{50}$ value of 2.59 ± 0.26 nM against HIV-1 subtype C K3016 virus.

The crystal structure of HIV-1 CA-SP1 Gag is a goblet-shaped hexamer where CA-CTD forms a cup, and the CA-SP1 junction lies within the stem of the six-helix bundle. The maturation inhibitors bind inside the hexamer cavity through hydrophobic and hydrogen bond interactions [16, 19]. In our study, maturation inhibitors with the same functional group but with longer lengths of linkers (2C and 3C linkers) were less active than compounds with 1C linkers. An increase in the length of carbon linker in the analogs may result in fewer and weaker interactions between HIV-1 Subtype C virus CA-SP1 Gag and maturation inhibitors.

A major problem during the clinical development of Bevirimat was that it was ineffective against HIV-1 viruses carrying polymorphism at the SP1 "QVT" region, specifically V7A. To develop broadly active drugs, it is imperative to test their activity against polymorphic viruses carrying V7A mutation. Hence, we checked the activity of these BVM analogs against the HIV-1 Subtype C K3016 virus, which has an inherent polymorphism in the QVT motif compared to the HIV subtype B NL4-3 virus. The K3016 virus has V7A and T8N natural polymorphism compared to the NL4-3 virus. One carbon linker compounds were potent against both HIV subtype B NL4-3 virus and Subtype C K3016 virus, suggesting that these compounds were broadly active against polymorphic viruses.

We have previously reported maturation inhibitors -CV-8611, CV-8612, and CV-8613, which had the same functional group as CV-10239, CV-10240, and CV-10238 but contained a 2C linker. These compounds were more potent than 3C linker compounds, confirming our findings that the minimal length of the linker provided optimal activity [14].

Interestingly, the compound GSK3640254, containing modifications at C-3 and C-28 positions of BVM, had lower $IC_{50}$ than CV-10240 [20]. Both compounds have the same functional group at the C-28 position, but GSK3640254 has an additional group at the C-3 position and lacks a linker at the C-28 position [17].

The BVM analogs retained activity against integrase inhibitor-resistant virus (NL4-3 11,847) and protease inhibitor-resistant virus (NL4-3 11,800) (Table 1), suggesting that these compounds could be not only be combined with other antiviral agents as part of the AIDS treatment regimen but also could be introduced if a patient developed resistance to existing drug treatment options.

## Conclusion

We have identified second-generation BVM analogs, which are broadly active against HIV-1 Subtypes B and C virus. The length of the carbon linker between C-28 carbon and the functional groups regulated their activity. The shorter the length of the linker, the better the activity of the maturation inhibitors. These studies will not only help in designing novel HIV maturation inhibitors with high efficacy and broad specificity but also suggest that the activity of previously reported MIs could be enhanced by reducing the length of the linker.

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12985-025-02635-8.

Supplementary file 1.

### Author contributions

Yuvraj KC: Conceptualization, Methodology, Validation, Formal analysis, Writing—original draft. Aradhana Singh: Methodology, Writing- Editing, and Review. Sayantani Datta, Ritika Das, Pranjal Raj Saxena, Subash Chapagain: Assistance in methodology. T.J. Nitz: Compound synthesis, review. Carl Wild: Compound synthesis, review. Ritu Gaur: Conceptualization, Methodology, Validation, Formal analysis, Writing—original draft, Review, Resources, Supervision, Funding acquisition.

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### Availability of data and materials

No datasets were generated or analysed during the current study.

## Declarations

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Competing interest

The authors declare no competing interests.

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## Publisher's Note