DPP9 INHIBITION ENHANCES NNRTI-MEDIATED CLEARANCE OF HIV-1 LATENT RESERVOIRS

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Introduction: We recently reported that non-nucleoside reverse transcriptase inhibitors (NNRTIs) can induce the caspase recruitment domain-containing protein 8 (CARD8) inflammasome in HIV-1 infected cells.[1] As seen in **Figure 1,** NNRTIs induce premature protease activation and subsequent cleavage of the N-terminus of autoprocessed CARD8, targeting it for N-terminal proteasomal degradation. The freed C-terminus can either induce the inflammasome – when high concentrations are present – or be inhibited by Dipeptidyl Peptidase 9 (DPP9).[2]

Due to the high binding affinity of human serum proteins for NNRTIs, highly effective intracellular concentrations may not be attained *in vivo*.[3] The DPP9 inhibitor Val-boroPro (VbP) can activate the CARD8 inflammasome in CD4⁺ T cells.[4,5] We therefore set out to identify if VbP can enhance NNRTI-mediated CARD8 inflammasome activation and the ability of VbP to elicit HIV-1-infected cell death on its own.

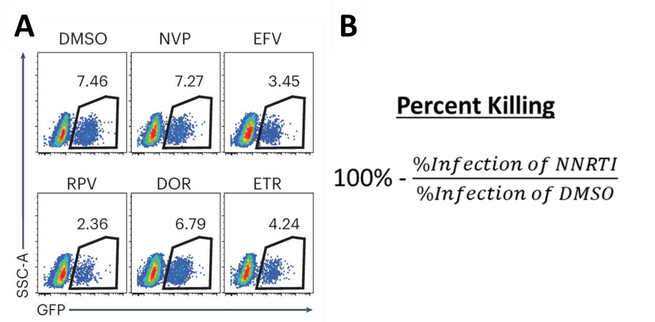


Figure 2: A) Representative gating of HIV infected CD4⁺ T cells treated with DMSO or NNRTIs. **B)** Calculation used to determine percent killing.

Methods: For all in vitro killing assays three donors of primary CD4⁺ T cells were isolated, stimulated with αCD3/CD28 antibodies, and infected with pNL4-3-pol-Δenv-GFP reporter virus, or reporter virus with NNRTI resistance mutations introduced via PCR. Three days later, NNRTIs, VbP, or DMSO were added in regular media or media containing 50% human serum for two days prior to assessment of infection rate via flow cytometry as seen in **Figure 2A**. Percent killing was calculated as shown in **Figure 2B.** For mouse experiments primary CD4⁺ T cells were isolated and infected as above then $1x10^7$ cells were transfused into the MISTRG-6-15 mouse model and mice were treated for 24 hours prior to tissue and blood collection. For IUPM measurement, CD4⁺ T cells isolated from people living with HIV (PLWH) were plated in limiting dilution and stimulated as above. Cells were treated for two days with NVP, EFV, or EFV + VbP. Cells were expanded and the infectious units per million (IUPM) was calculated to identify reductions in viral reservoir size due to VbP treatment. Data analysis was done using Flowjo and GraphPad Prism.

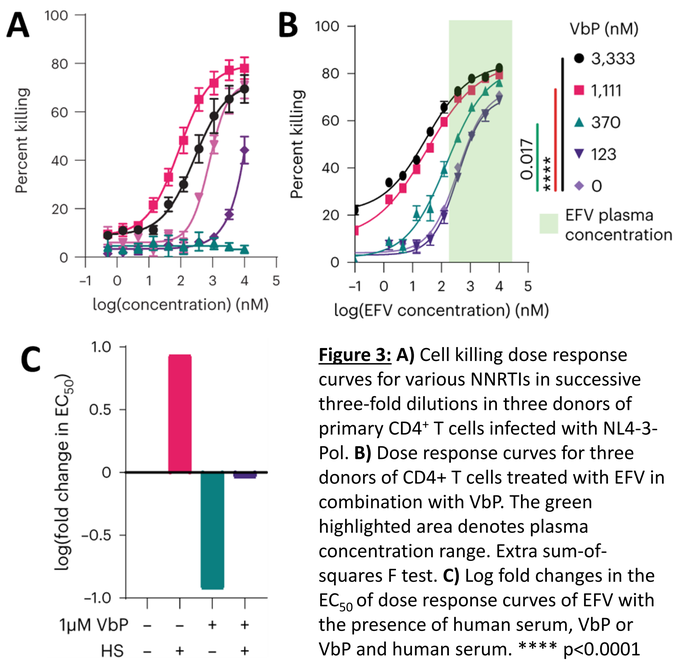


Figure 3: A) Cell killing dose response curves for various NNRTIs in successive three-fold dilutions in three donors of primary CD4⁺ T cells infected with NL4-3-Pol. **B)** Dose response curves for three donors of CD4+ T cells treated with EFV in combination with VbP. The green highlighted area denotes plasma concentration range. Extra sum-of-squares F test. **C)** Log fold changes in the EC_{50} of dose response curves of EFV with the presence of human serum, VbP or VbP and human serum. **** p<0.0001

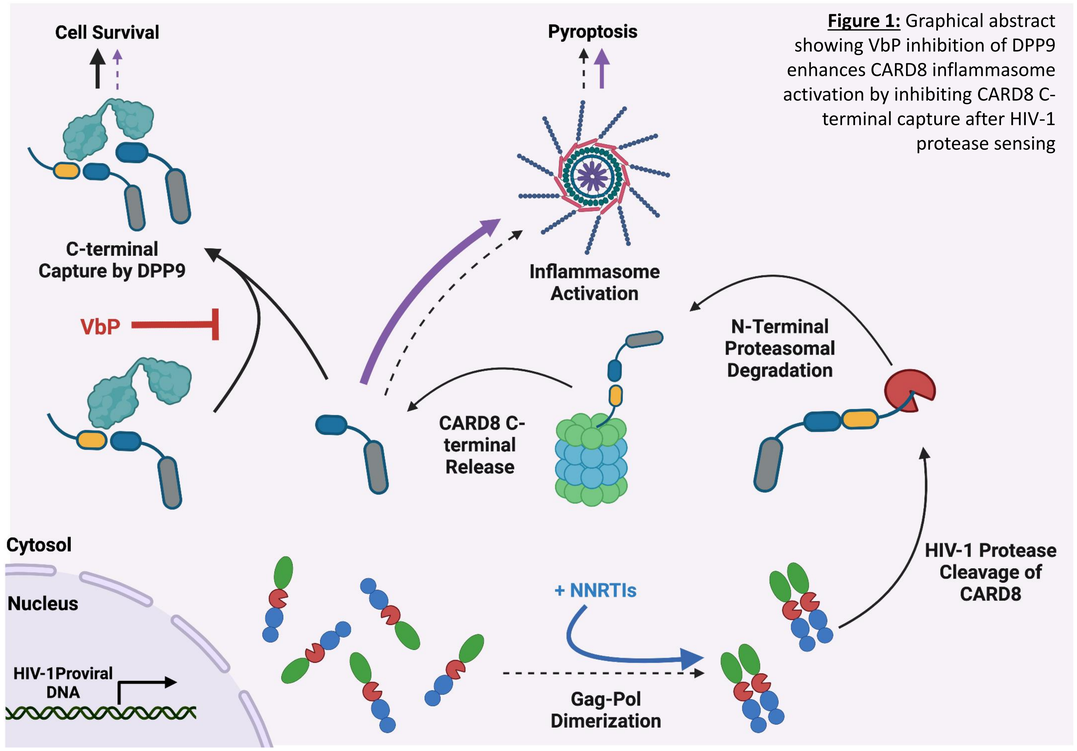
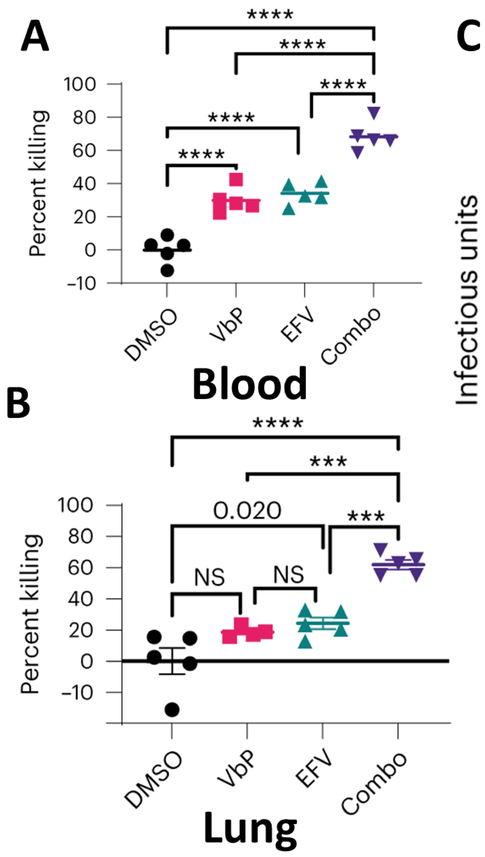


Figure 1: Graphical abstract showing VbP inhibition of DPP9 enhances CARD8 inflammasome activation by inhibiting CARD8 C-terminal capture after HIV-1 protease sensing



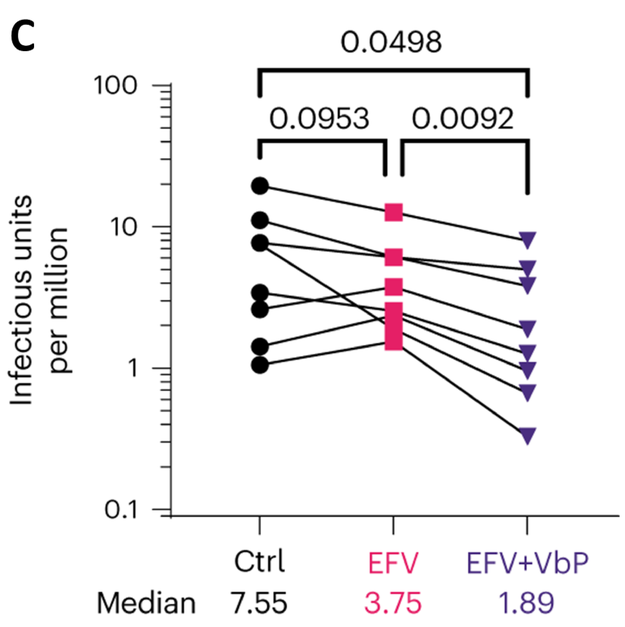


Figure 4: A and B) Killing of infected human CD4⁺ T cells transfused into the MISTRG-6-15 mouse model 24hr post treatment in a) blood or b) lung. One-way ANOVA with Tukey's multiple hypothesis test. **C)** Infectious units per million of CD4⁺ T cells isolated from PLWH and treated with NVP, EFV, or EFV + VbP. Median IUPM values are listed below the graph. One-way ANOVA with Holm–Šídák's multiple comparisons test. **** p<0.0001, ***p<0.001

Results: To understand the potential utility of using NNRTIs as a "shock and kill strategy" we first assessed the killing efficiency at clinically relevant concentrations (**Figure 3A**).[6,7] NNRTIs known binding affinity for human serum proteins highlights the need for an enhancement of NNRTI induction of pyroptosis. Utilizing the DPP9 inhibitor VbP, we were able to show that blocking DPP9's ability to inhibit the CARD8 inflammasome increases its sensitivity to triggers like HIV-1 protease (**Figure 3B**). It is also of note that VbP can eliminate HIV-1-infected CD4⁺ T cells without the presence of NNRTIs. We also show that VbP can ameliorate issues of human serum binding and bring the EC_{50} of killing by EFV back to baseline (**Figure 3C**). We then tested this strategy *in vivo* and showed that EFV or VbP alone were able to kill HIV infected cells in the MISTRG-6-15 mouse model which was greatly improved with combination treatment as evidenced in blood and lung tissue (**Figure 4A and B**). To further demonstrate the clinical relevance of this approach, we demonstrated that VbP treatment can further promote clearance of HIV-1 reservoirs isolated from PLWH as evidenced by reductions in the IUPM values of combination treated cells (**Figure 4C**). Finally, we show that VbP sensitization can help address reductions in NNRTI efficacy due to resistance mutations (**Figure 5**).

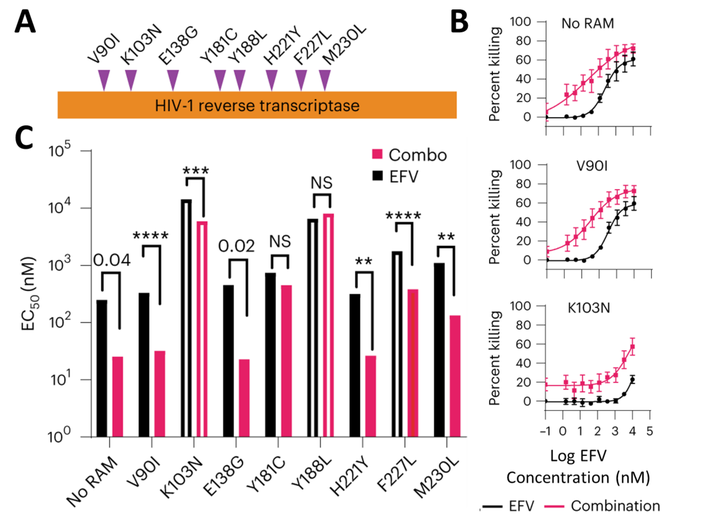


Figure 5: A) Graphical depiction of the location of NNRTI RAMS. **B)** Dose response curves for the selected NNRTI RAMs CD4⁺ T cells treated with serial three-fold EFV dilutions with (red) or without (black) the presence of 1μM of VbP**. C)** EC_{50} values are shown for all viruses with or without the presence of VbP. Significance was calculated using extra sum-of-squares F test. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001

Conclusions: Since NNRTIs offer a promising strategy for eradication of HIV-1 latent reservoirs, improving their in vivo cell killing potency is essential to treatment efficacy. This study proves that sensitization of the CARD8 inflammasome through DPP9 inhibition, *in vitro* and *in vivo*, can reduce the threshold of CARD8 activation and provide more effective clearance of HIV infected cells for clinically relevant scenarios. This phenotype held true *in vivo* in humanized mice and *ex vivo* via the elimination of HIV viral reservoirs from PLWH. We also show that VbP can reduce the negative effects on killing due to NNRTI resistance mutations and bring the killing efficacy back to comparable levels as the control. VbP was also shown to kill HIV-1-infected cells without the presence of NNRTIs. This provides a new class of drugs as potential candidates for therapeutics that selectively kill HIV-1 infected cells, highlighting the need for further investigation and screening of new DPP9 inhibitors with greater specificity and less toxicity than VbP.



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