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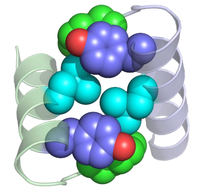
SYNTHESIS AND EVALUATION OF TIGHT-BINDING HYDROXYPYRAZOLE INHIBITORS OF HIV-1 NEF

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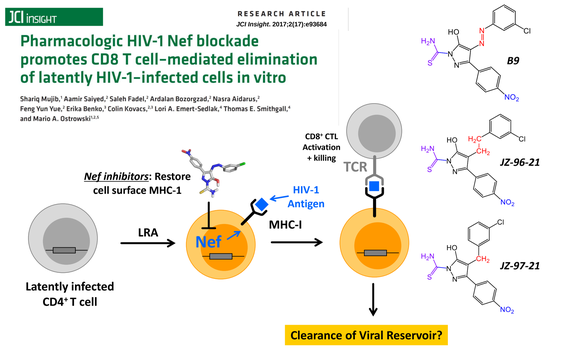
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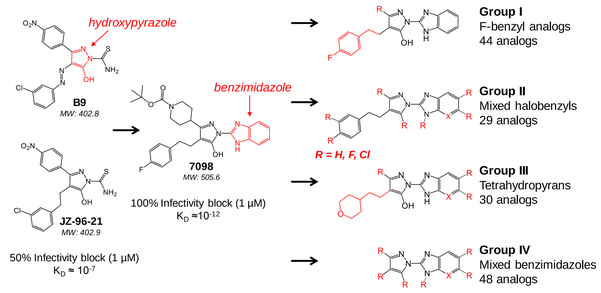


Introduction

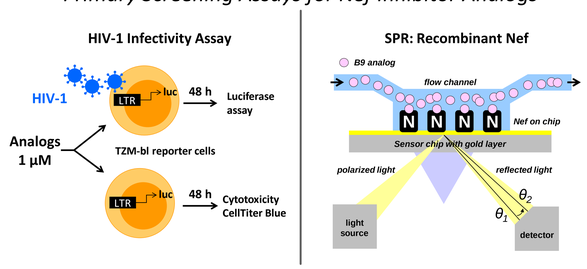
- Nef is an HIV-1 accessory factor critical for viral replication, immune evasion and AIDS progression
- Nef lacks intrinsic biochemical functions and hijacks host cell signaling and trafficking pathways for the benefit of the virus
- HTS for inhibitors of Nef-dependent Src-family kinase activation enabled discovery of small molecule Nef inhibitors (hydroxypyrazoles)
- Hydroxypyrazole inhibitors bind directly to Nef and block multiple Nef functions related to infectivity, replication, and MHC-I downregulation
- Nef inhibitors in this class promote CTL-mediated elimination of latently infected CD4⁺ T cells in vitro



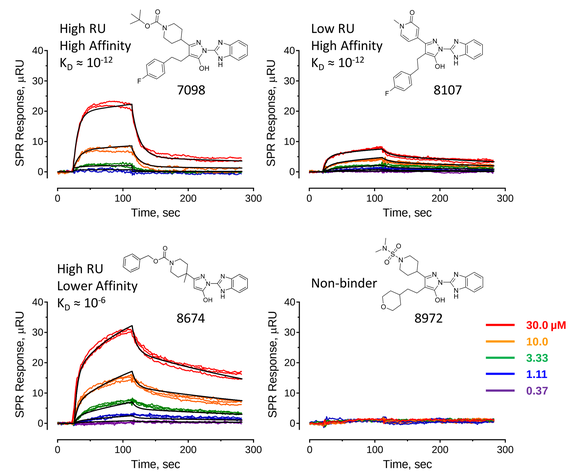
Second-generation hydroxypyrazole Nef Inhibitors



Primary Screening Assays for Nef Inhibitor Analogs

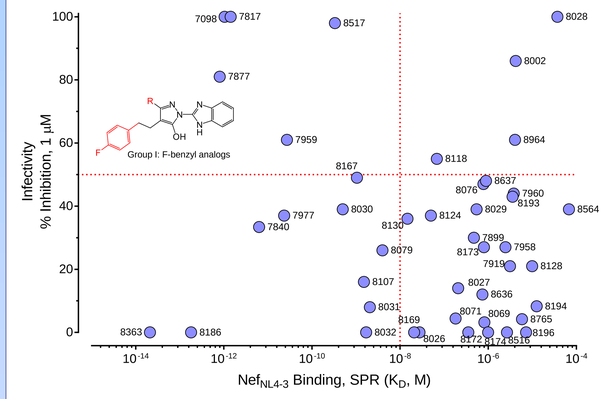


Representative SPR behavior of inhibitor analogs



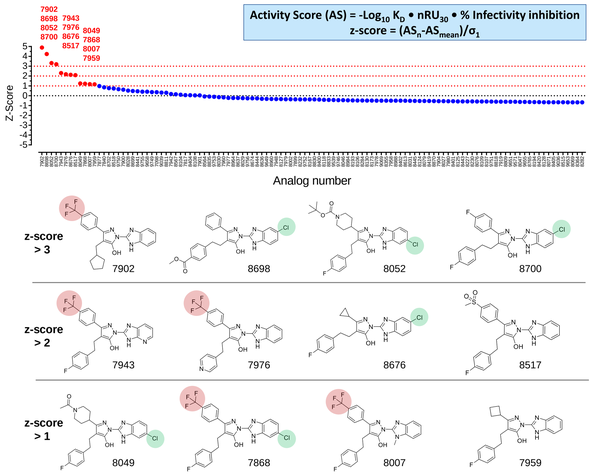
Analysis of Nef interaction with hydroxypyrazole inhibitors by surface plasmon resonance (SPR). Recombinant HIV-1 Nef protein (NL4-3) was expressed in *E. coli* and purified to homogeneity. The Nef protein was immobilized on the surface of a carboxymethyl dextran hydrogel biosensor chip, and each analog was injected over the range of concentrations shown in triplicate. The resulting sensorgrams were best-fit by a two-step induced-fit binding model, and the K_D values shown were calculated from the resulting kinetic rate constants. All data were recorded on a Reichert 4-Channel SPR instrument using Reichert biosensor chips. Four general types of binding behavior were observed, and representative examples of each are shown for the indicated analogs.

Infectivity vs. affinity plot: Group I Analogs



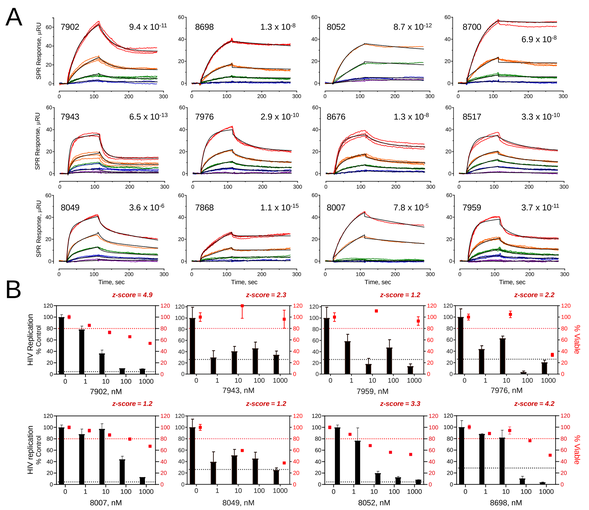
Assessment of Nef inhibitor analog efficacy in infectivity assays and by SPR. Antiretroviral activity of each analog was assessed in TZM-bl reporter cells in which the HIV-1 LTR drives expression of luciferase. Cells were infected with HIV-1_{NL4-3} in the presence of each compound at a final concentration of 1.0 μM, and luciferase activity was determined 48 h later. Cell viability in the presence of each compound was assessed in the absence of virus using the CellTiter-Blue assay (*data not shown*). Cells infected with wild-type and Nef-deleted HIV-1 in the absence of compounds served as controls and defined the signal window. All assays were performed in triplicate, and the data are expressed as percent inhibition relative to the plate controls. Each compound was also assessed for direct interaction with Nef by SPR, and K_D values were calculated from the resulting kinetic constants (*see adjacent panel*). Percent infectivity inhibition is plotted vs. K_D, and arbitrary cut-offs of 50% inhibition and K_D = 100 nM are shown for reference (*dotted red lines*). This approach does not provide a rank order of activity nor does it incorporate the extent of binding, which varies widely between the compounds. Data for inactive compounds are not shown.

Activity Score ranking for all benzimidazole inhibitor analogs



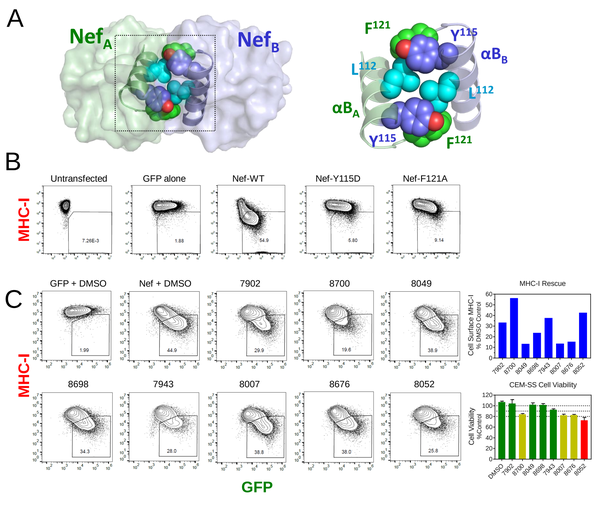
Integration of SPR and infectivity data enables ranking of Nef inhibitor analog activity. Factors combined to define an 'Activity Score' for each inhibitor analog include affinity, extent of binding, and antiretroviral activity. K_D values were determined by SPR and the -Log of the K_D provides a range of values from 5 to 15 (corresponding to 10 μM to 0.01 pM). The extent of binding is based on SPR response units (RU) observed at the highest concentration tested (30 μM) and normalized to the amount of Nef protein immobilized on each chip (RU_{Nef}) such that $nRU_{30} = RU_{30}/RU_{Nef}$ (values range from 6.4 x 10^{-4} to 4.3 x 10^{-2}). Percent infectivity inhibition is determined in the TZM-bl assay relative to the wild-type and ΔNef HIV-1 controls. A z-score was then calculated for each compound relative to the mean of all non-zero Activity Scores. Of 146 analogs tested, 114 were active and used in the z-score analysis shown above; structures of compounds with z-scores > 3, > 2 and > 1 are shown below the plot.

SPR Sensorgrams and anti-retroviral activity of top analogs



Top scoring Nef inhibitor analogs potently inhibit HIV-1 replication in donor PBMCs. *A*) SPR sensorgrams for the 12 most active compounds out of 146 analogs tested. *B*) Antiretroviral activity was assessed in donor PBMCs infected with HIV-1_{NL4-3} over the range of inhibitor concentrations shown. HIV-1 p24 was assessed by AlphaLISA assay (PerkinElmer) after 4 days in culture. Cytotoxicity was independently assessed using the CellTiter-Blue assay (Promega). Each plate included control wells infected with wild-type vs. ΔNef HIV-1 in the absence of inhibitor. The black dotted line shows the level of HIV-1 ΔNef replication in each experiment. All data are shown as mean percent control (no inhibitor) ± SE. Compounds **8676** and **8700** were inactive at non-toxic concentrations (not shown); **7868**, **8517** not tested.

Inhibition of Nef-mediated MHC-I downregulation



Nef homodimerization mutants and inhibitors both reverse MHC-I downregulation. *A*) Crystal structure of the HIV-1 Nef dimer interface (PDB: 1EFN). Overall structure (left); α-helical core enlarged on the right. Conserved residues in this region include Leu112, Tyr115, and Phe121. *B*) Nef homodimer interface mutants are defective for MHC-I downregulation. The CEM-SS T cell line, which is engineered to over-express an HLA-A2 allele, was transfected with an expression plasmid for HIV-1 Nef plus GFP as a gating marker. After 48 h, cells were fixed and stained for cell-surface HLA-A2 and analyzed by flow cytometry. The percentage of GFP⁺ cells showing downregulation of MHC-I are shown. *C*) Effect of Nef inhibitors on Nef-dependent MHC-I downregulation (tested at 1 μM; cell viability assessed by CellTiter Blue assay).

Summary and Conclusions

- HIV-1 Nef inhibitors based on a hydroxypyrazole core are amenable to a wide range of structural modifications and retain potent inhibitory activity despite addition of bulky heterocyclic substituents
- Integration of infectivity and SPR data as a single numerical Activity Score for each analog enabled ranking across analogs to prioritize further evaluation and development
- Activity Scores were predictive of antiretroviral activity in donor PBMCs and reversal of MHC-I downregulation
- Nef inhibitors and mutations of the Nef homodimerization interface both impact MHC-I downregulation, suggestive of a shared mechanism of action affecting Nef quaternary structure
- Ongoing studies are evaluating pharmacologic properties *in vivo* with the goal of identifying analogs suitable for testing in humanized mouse models of HIV-1 replication and latency

References

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