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Purinergic Receptor Antagonists as Potent Inhibitors of HIV-1 Infection by Blocking Early Entry

Talia H. Swartz[1], Anthony Esposito[1], Natasha Durham[1], Benjamin K. Chen[1]

[1]*Division of Infectious Diseases, Department of Medicine, Icahn School of Medicine at Mount Sinai, New York, NY*

talia.swartz@mssm.edu



Abstract

Human Immunodeficiency Virus (HIV-1) causes an incurable infection that simultaneously induces immune cell depletion and activation of inflammatory pathways. Although drugs can largely suppress the viral replication, the activation of inflammatory pathways remains a cause of morbidity. Recent studies have proposed that ATP (adenosine triphosphate) release and purinergic signaling is required for HIV-1 viral infection. Purinergic receptors are distributed through a wide variety of tissue types that detect extracellular ATP as a danger signal released from dying cells. Given this important role in immune cell signaling and viral infection, we have explored how these pathways are involved in transmission of HIV-1 virus from cell to cell through virological synapses. Our laboratory has devised molecular tools to study virus-host interactions through the use of fluorescent tags, allowing us to visualize discrete stages of infection. Using these infection assays, we are able to determine the step at which purinergic antagonists block cell-cell infection. We have also developed a Cre-recombinase-driven assay to detect viral fusion only in target cells following virological synapse formation We have found that purinergic antagonists can robustly inhibit HIV-1 infection through cell-cell contact in a dose-dependent manner, and that this inhibition does not affect the formation of virological synapses or the subsequent cell-cell transfer of HIV-1. We further determined that purinergic inhibition inhibits viral membrane fusion. We are currently examining whether purinergic signaling is required for the recruitment of the viral co-receptors to the synapse to support viral fusion. We foresee broad implications for this pathway in the understanding of HIV-1 virology as well as the pathogenesis of long-term sequelae that underlie chronic HIV-1 infection. We anticipate that drug discovery in this area may yield a therapeutic target with both potent antiviral and anti-inflammatory properties.

Introduction

Literature has emerged supporting the role of extracellular ATP and purinergic signaling in HIV-1 transmission. Evidence from confocal microscopy has suggested that the purinergic receptor and downstream targets may co-localize at the cell-cell interface, the so-called virological synapse where virus is transferred from target cell to host cell (Figure 1, 2). Our laboratory has developed techniques through labeling of virus that have been used to elucidate the concept of cell-cell transmission of HIV-1 because previously it has been thought that virus spread occurred in a cell-free modality. Based on this paradigm, we assessed whether inhibition of purinergic receptor signaling would result in abrogation of cell-cell transmission of HIV.

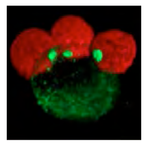


Figure 1. Confocal microscopy of HIV-1 transfer through virological synapse, 3D reconstruction. HIV Gag-iGFP-expressing Jurkat T cell (green) synapsed with three primary CD4 T cells [red, labeled with CellTracker Orange CMRA (Invitrogen, Carlsbad, CA)].

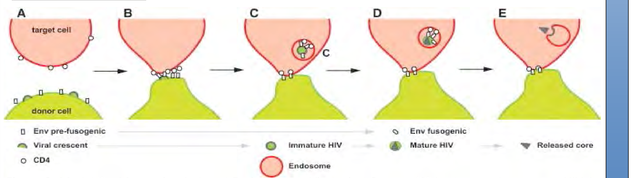


Figure 2. Model for Maturation-Induced Fusion at the Virological Synapse(A) In the steady state, an HIV-1 infected T cell has a diffuse distribution of Env and Gag.(B) The engagement of Env on the infected donor cell, with CD4 on the target cell, induces an adhesion event and results in the recruitment of Gag, Env, and CD4 to the VS.(C) An endocytic event is triggered resulting in CD4-dependent uptake of immature virus into acceptor cell intracellular compartments.(D) Bound to CD4, virus particles undergo protease-dependent maturation over time. (E) Viral particle maturation triggers viral membrane fusion, releasing the capsid to the cytoplasm.

Methods

Viral Constructs. HIV-1 NL-GI contains GFP in place of nef, and nef is expressed from a downstream internal ribosome entry site (IRES). Primary CD4+ T-cells were infected with NL-GI containing the NL4-3 envelope (X4-tropic) or the R5-tropic B-clade primary envelope from pRHPA4259 clone 7 (SVPB14), obtained through the AIDS Research and Reference Reagent Program (ARRP), Division of AIDS, NIAID, NIH from Drs. B. H. Hahn and Dr. J. F. Salazar-Gonzalez. Gag-iCre was generated with a Cre-recombinase inserted as fusion in gag polypeptide.

Cells and cell culture. Human cell lines Jurkat E6-1 and MT4 (CXCR4, Arthur Weiss and Douglass Richman respectively, ARRP) are maintained in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 g/mL streptomycin and 2 mM glutamine (complete RPMI).

Inhibitors. A panel of inhibitors were tested for the ability to block cell-free and VS-mediated infection using 5-fold serial dilutions beginning at the given concentration, unless otherwise stated. This includes: 50 µM Pyridoxal phosphase-6-azo(bendene2-4-D, PPADS) (Sigma), a non-selective P2 antagonist. A library of 71 purinergic antagonists and agonists (Enzo BML-2820 v 4.3) was used for the high-throughput fusion assay.

Cell-free infection assay. Cell-free virus particles were produced in 293T cells by calcium-phosphate transfection. Viral supernatants are quantified by p24 ELISA. MT4 cells are infected in 96-well plates with sufficient NL-GI to obtain up to 10% infection after 48 hrs in the absence of inhibitors. Virus supernatant and MT4 cells were pre-incubated separately with equal volumes of inhibitors for 30 min at 37ºC before mixing. After 18 hrs, culture media is replaced with complete RPMI containing 10 µM zidovudine. 48 hrs after mixing, cells are treated with trypsin and fixed in 2% paraformaldehyde.

VS-mediated infection assay. Jurkat (donor) cells are transfected by nucleofection (Amaxa Biosystems) with 5 µg NL-GI DNA, cultured overnight in antibiotic-free medium and purified by Ficoll-Hypaque density gradient centrifugation. Jurkat cells and MT4 (target) cells are dye-labeled with 1 µM CellTrace Far Red DDAO-SE (Molecular Probes) for 10 min at 37°C, or 20 µM CellTracker Blue CMF_2HC (Molecular Probes) for 30 mins at 37°C, respectively. 0.125 x 10^6 donor or target cells are pre-incubated separately with inhibitors for 30 mins at 37ºC before mixing at a ratio of approximately 1:1, co-cultured at 37ºC for 36 hrs, treated with trypsin and fixed.

VS-mediated transfer assay. Jurkat (donor) cells are transfected by nucleofection (Amaxa Biosystems) with 5 µg iGag-GFP DNA, cultured overnight in antibiotic-free medium and purified by Ficoll-Hypaque density gradient centrifugation. Jurkat cells and MT4 (target) cells are dye-labeled with 1 µM CellTrace Far Red DDAO-SE (Molecular Probes) for 10 min at 37°C, or 20 µM CellTracker Blue CMF_2HC (Molecular Probes) for 30 min at 37°C, respectively. 0.125 x 10^6 donor or target cells are pre-incubated separately with inhibitors for 30 mins at 37ºC before mixing at a ratio of approximately 1:1, co-cultured at 37ºC for 4 hrs, treated with trypsin and fixed.

Fusion assay. A stable cell line of Jurkats was generated with using an MSCV vector expressing a dsRed reporter flanked by loxP sites (RG = red-green). Jurkat (donor) cells are transfected by nucleofection (Amaxa Biosystems) with 5 µg Gag-iCre DNA, cultured overnight in antibiotic-free medium and purified by Ficoll-Hypaque density gradient centrifugation. 0.125 x 10^6 donor or target cells are pre-incubated separately with inhibitors for 30 mins at 37ºC before mixing at a ratio of approximately 1:1, co-cultured at 37ºC for 48 hrs, treated with trypsin and fixed.

Results

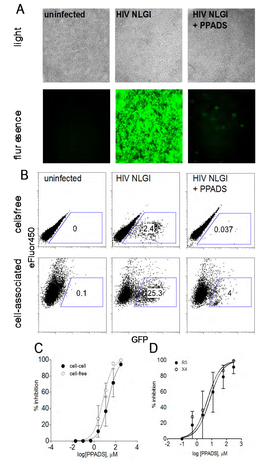


Figure 3. Cell-Cell and Cell-Free Infection of HIV-1 Is Blocked by Purinergic Receptor Inhibition. The T cell line MT4 was incubated with Jurkat cells expressing HIV-1 NL-GI and pre-incubated in the +/- PPADS. (**A**) Cell-free viral infection is inhibited by PPADS, bright field (top) and fluorescence (bottom) images of cells infected with cell-free NL-GI in +/- PPADS. (**B**) Cell-free (top) and Cell-cell (bottom) are both inhibited by 100 µM PPADS as measured by flow cytometry. (**C**) Dose response curve of both cell-cell and cell free infections by serial 5-fold dilutions of 100 µM PPADS, non-linear regression curve fit shown. (**D**) PPADS blocks productive infection for cell-free virus with X4- and R5-tropic virus infecting MT4 cells and MT4 cells expressing CCR5, respectively.

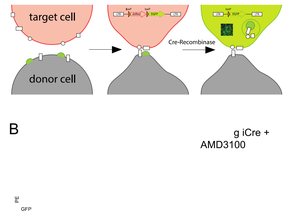


Figure 4. PPADS inhibits HIV fusion using a novel assay using an HIV Cre-recombinase virus. (**A**) Model for fusion assay in which a gag-iCre construct is transfected into Jurkat cells that as donor cells and are mixed with target Jurkat cells carry an integrated recombination substrate that constitutively expresses DSred and are activated by Cre to express GFP following fusion of Cre-carrying viruses with the target cell. (**B**) Representative flow cytometry plots of VS-mediated, viral membrane fusion assay, with GagiCre transfected Jurkat cells mixed with DS-red expressing target cells after 48 hours with AMD3100 and increasing concentrations of PPADS.

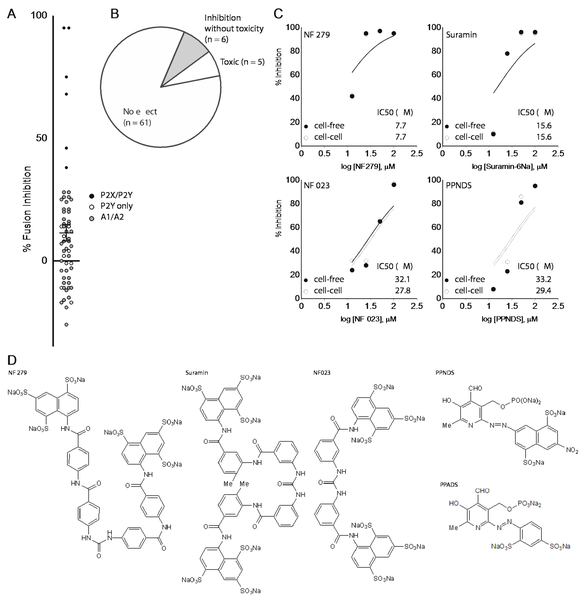


Figure 5. Screen of purinergic library reveals that compounds selective for P2X receptors inhibit viral membrane fusion. A. Dot plot of all compounds plotted based on inhibition in Cre-Lox assay. B. All compounds are listed, 6 had inhibitory activity without toxicity while 5 compounds had inhibitory activity at a toxic level. The remaining 61 compounds had no effect. C. Dose titrations were conducted using the Cre-Lox assay in cell-free and cell-cell fusion. IC_{50}s were calculated using non-linear regression and listed for each compound. D. The 5 compounds that were most inhibitory and their structures are shown.

Conclusions

We demonstrate that purinergic inhibition by PPADS has a potent inhibitory effect on HIV infection in both cell-cell and cell-free systems (Figure 3). This appears to be acting at the level of viral membrane fusion (Figure 4-5) and is specific for P2X receptor signaling. A model for purinergic signaling and its impact on HIV infection is shown in Figure 6.

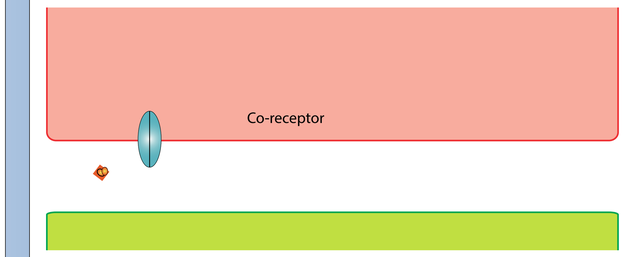


Figure 6. Model for purinergic signaling in early HIV cell-cell infection. A donor cell is shown in close contact with a target cell at the virological synapse. HIV Env binds to CD4 that may trigger ATP release through pannexin (the ATP channel). This extracellular ATP is detected by the purinergic receptor (denoted as P2), which may serve to recruit coreceptor to the site of HIV entry, thus allowing membrane fusion. Downstream events such as inflammasome activation and pyroptosis may subsequently occur as a result of these events.

We show that P2X receptor inhibitors specifically inhibit HIV productive infection at the level of fusion and describe an assay for high-throughput screening of inhibitors of HIV fusion. Our results suggest a potent novel target for HIV therapeutics. Future directions to this project will be to investigate the mechanism by which purinergic inhibition affects HIV entry and to evaluate the downstream targets of purinergic signaling and how these impact on HIV entry. We will investigate the role of inflammasome activation in HIV infection. Activation of this pathway would suggest significant implications for understanding the fundamentals of HIV-infected T cell biology as well as identifying novel therapeutic targets in HIV disease. We foresee broad implications for this pathway in the understanding of HIV virology as well as the pathogenesis of long-term sequelae that underlie chronic HIV infection. We anticipate that drug discovery in this area may yield a therapeutic target with both potent antiviral and anti-inflammatory properties.

References

1. Barat C, Gilbert C, Imbeault M, Tremblay MJ. Extracellular ATP reduces HIV-1 transfer from immature dendritic cells to CD4+ T lymphocytes. Retrovirology 2008;5:30.
2. Chen P, Hubner W, Spinelli MA, Chen BK. Predominant mode of human immunodeficiency virus transfer between T cells is mediated by sustained Env-dependent neutralization-resistant virological synapses. J Virol 2007;81:12582-95.
3. Durham ND, Yewdall A, Chen P, et al. Neutralization resistance of HIV-1 virological synapse-mediated infection is regulated by the gp41 cytoplasmic tail. J Virol 2012; Epub ahead of print.
4. Guaraldi G, Orlando G, Zona S, et al. Premature age-related comorbidities among HIV-infected persons compared with the general population. Clin Infect Dis 2011;53:1120-6.
5. Jolly C, Kashefi K, Hollinshead M, Sattentau QJ. HIV-1 cell to cell transfer across an Env-induced, actin-dependent synapse. J Exp Med 2004;199:283-93.
6. Seror C, Melki MT, Subra F, et al. Extracellular ATP acts on P2Y2 purinergic receptors to facilitate HIV-1 infection. J Exp Med;208:1823-34.