Poster Number

H-930

49th Interscience Conference on
Antimicrobial Agents and Chemotherapy (ICAAC)
September 12-15, 2009
San Francisco, CA USA

HIV Integrase Inhibitors Do Not Exert A Post-Antibiotic Effect Despite Slow Dissociation From IN-DNA Complexes *In Vitro*

R Hluhanich, A Kinkade, NA Margot, M Tsiang, R Geleziunas, M Wang, MD Miller, and DJ McColl

Gilead Sciences, Inc., Foster City, CA, USA

GILEAD
Gilead Sciences, Inc.
333 Lakeside Drive
Foster City, CA 94404
Tel: (650) 522-5821
Fax: (650) 522-5890

Introduction

- Post-antibiotic effect (PAE) is a delayed resumption in bacterial growth noted for some antibacterials, e.g., aminoglycosides, when drug concentrations fall below the minimum inhibitory concentration[1]
- PAE is determined by *in vitro* growth kinetics and has been used to justify dosing adjustments of some antibacterials *in vivo*
- Once-daily (QD) dosing of HIV-1 antiretroviral (ARV) drugs can be achieved due to their intrinsic pharmacokinetic (PK) properties
  - Long plasma half-life ($t_{1/2}$ > 24 h) of the NNRTI efavirenz
  - Intracellular $t_{1/2}$ >24 h of the activated NRTI tenofovir-diphosphate
  - Boosting via CYP3A inhibition with ritonavir (Boosted PIs)
- Robust PK of ARVs, such as a C_{min} concentration above the protein adjusted EC_{95}, is considered necessary to inhibit HIV-1 replication and prevent emergence of HIV-1 drug resistance
- The potential of PAE to impact dosing of ARVs, such as the integrase inhibitors (INIs), has not been established

Background

- The HIV-1 INI raltegravir (RAL, Isentress) is approved for twice-daily (BID) dosing based on its plasma PK and RAL cannot be boosted to QD dosing with ritonavir (RTV)
- The INI elvitegravir (EVG), currently in phase 3 trials, can be boosted using RTV or a novel booster, GS-9350, allowing QD dosing of EVG[2]
- A post-antibiotic effect has been proposed for the INI RAL[3]
- The capacity of the INIs RAL and EVG, to mediate effects analogous to PAE was investigated

Objectives

- Verify the slow dissociation of INIs from IN-DNA complexes
- Investigate whether effects analogous to PAE can be demonstrated for the HIV-1 INIs RAL and EVG, and other ARVs *in vitro*
- Investigate the intracellular antiviral persistence of RAL and EVG *in vitro*
- Investigate the capacity of INIs to block HIV infection under *in vitro* conditions that mimic asynchronous infections in HIV patients

Methods

Binding and Dissociation of INIs from IN-DNA Complexes

- Biotinylated donor DNA (360 nM) was bound to streptavidin-coated PVT SPA beads for 1 h at 25ºC. Unbound donor DNA was removed and beads were bound to 1.5 μM IN for 1 h at 25ºC. The bead-IN complex was dispensed to white 96 well plates and [^{3}H]-INI was added. Binding proceeded at 25ºC until equilibrium was reached. Excess unlabeled INI (5 μM) was added. Dissociation at 25ºC was measured on a TopCount each min continuously for > 72 h as described by Grobler et al.[4] INI binding and dissociation data were fitted to a two-step binding model for calculation of K_{off}, as described by Langley et al.[5]

Time of Addition Experiments

- Cells were infected with HIV-1_{IIIB} at MOI 0.1 for 2 h, then virus was removed. Infected cells were exposed to INIs (RAL, 1.25 μM; EVG, 250 nM; GSK364735, 500 nM; or GS-9160, 500 nM) at 2, 6, 8, 10, 12, 15, or 24 h post infection. Supernatant (SNT) was harvested 48 h post-infection and p24 was measured

Intracellular Drug Persistence Assays

- Cells were exposed to RAL, EVG, or LPV at 500xEC_{50} or TFV at 100xEC_{50} for 15 h at 37ºC. Drug was removed by washing cells and cells were infected with HIV-1_{IIIB} at MOI 0.3 for 3 h. Virus was removed and SNT was harvested 48 h later and p24 was measured

Wash Out Time-Courses: "Post-Antibiotic Effect" Assays

- Cells were infected with HIV-1_{IIIB} at MOI 0.3 for 2.5 h. Virus was removed and the infected cells were exposed to RAL, EVG, or LPV at 500xEC_{50} or TFV at 100xEC_{50}. Drug was removed at 6, 12 and 24 h post-infection. SNT was harvested 48 h post-infection and p24 was measured. For extended time-courses, cells were infected with HIV-1_{IIIB} at MOI 0.3 for 2.5 h. Virus was removed and infected cells were exposed to RAL, EVG, or EFV at 500xEC_{50} or TFV at 100xEC_{50}. Drug was removed 24 h post-infection. SNT was harvested 48, 72 and 96 h post infection and p24 was measured

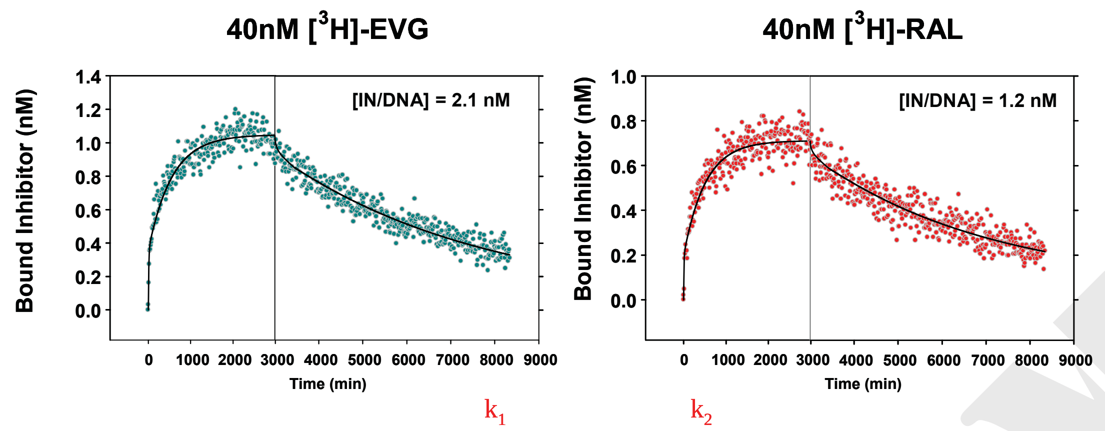
Methods (cont'd)

Rechallenge of Cells with Firefly Luciferase (FF-Luc) and Renilla Luciferase (Ren-Luc) Viruses

- The *nef* gene of HIV-1_{pLAI} proviral DNA was replaced with either the Firefly or Renilla luciferase gene to create the respective FF-Luc and Ren-Luc reporter viruses. Cells were infected with HIV-1_{pLAI} FF-Luc reporter virus for 3 h. RAL, EVG or EFV were added at free fraction adjusted C_{max} and TFV was added at 100xEC_{50}. Infected cells and drug were incubated for 24 h post infection. Virus and drug were removed and cells were reinfected with HIV-1_{pLAI} Ren-Luc reporter virus at MOI 0.08 under conditions of drug titration. Drug titrations were serial dilutions from a C_{max} free fraction equivalent, derived by equilibrium dialysis of each drug with plasma versus dialysis with cell culture medium. Both FF and Ren luciferase signals were measured at 72 h after FF-Luc infection (48 h post Ren-Luc infection) using Dual Glo (Promega)

Results

Figure 1. Both EVG and RAL Dissociate Slowly from IN-DNA Complexes with Similar K_{off} Rates

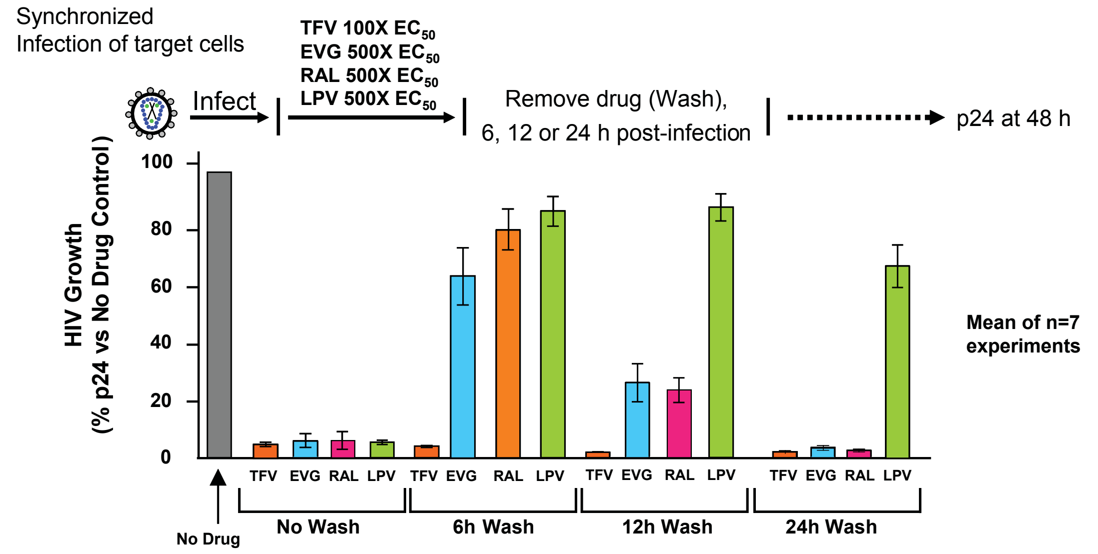


$$E + L \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} EL \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} EL_f$$

	k_1, $\mu M^{-1}s^{-1}$ (x 10^{-3})	k_{-1}, s^{-1} (x 10^{-4})	k_2, s^{-1} (x 10^{-4})	k_{-2}, s^{-1} (x 10^{-4})	k_{on}, $\mu M^{-1}s^{-1}$ (x 10^{-3})	k_{off}, s^{-1} (x 10^{-4})	Calculated $t_{1/2}$, hour
^{3}H-RAL	5.5	9.2	1.1	0.2	0.7	0.18	11.0
^{3}H-EVG	8.2	6.7	0.8	0.2	1.1	0.17	11.1

- RAL and EVG have similar slow K_{off} rates, but does this mediate a post-antibiotic effect?

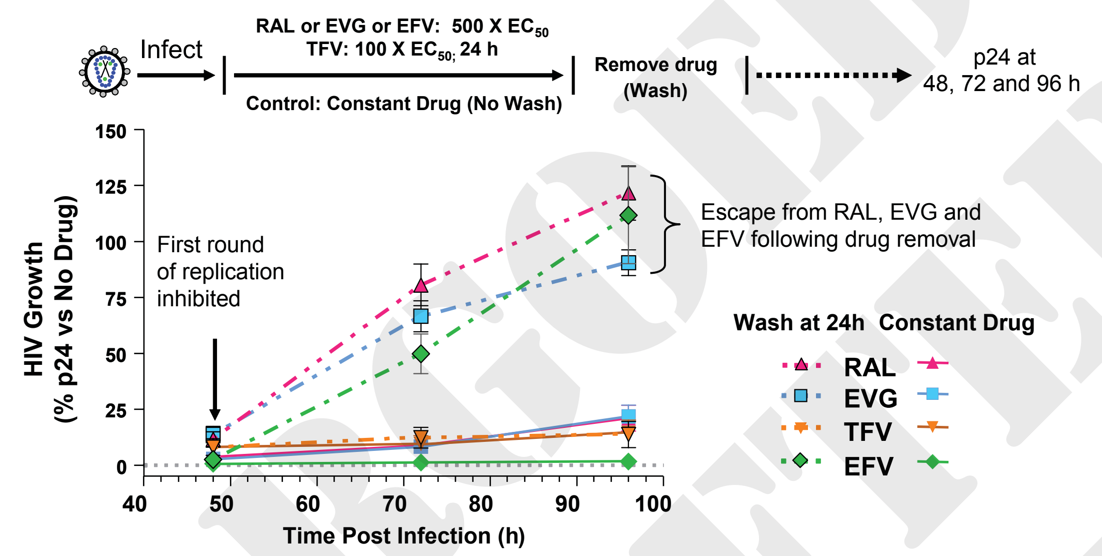
Figure 2. Removal of INIs Affects Viral Replication in a Time-Dependent Fashion



- Removal of drugs post infection results in inhibition of the viral lifecycle at the expected time for each drug
  - Removal of INIs at 6 h post-infection minimally inhibited replication
  - Most INI activity occured by 12 h post-infection but some infections escaped inhibition
  - Removal of INIs at 24 h showed full inhibition of infection as integration blockage was complete for a synchronous single round of infection

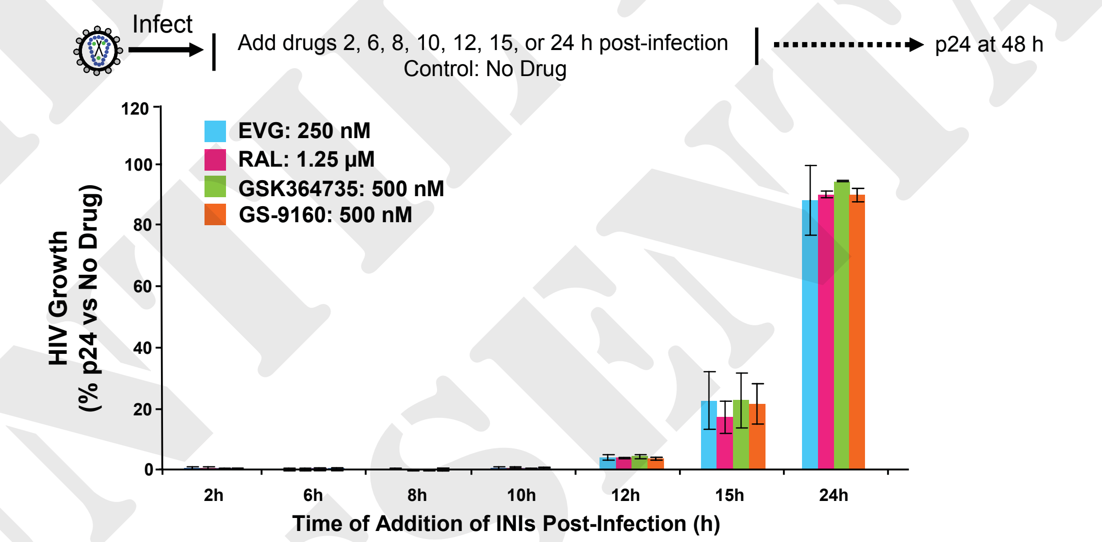
Results (cont'd)

Figure 3. HIV Escape from INIs but not TFV, Following Drug Removal, Indicates Lack of a Post-Antibiotic Effect by INIs



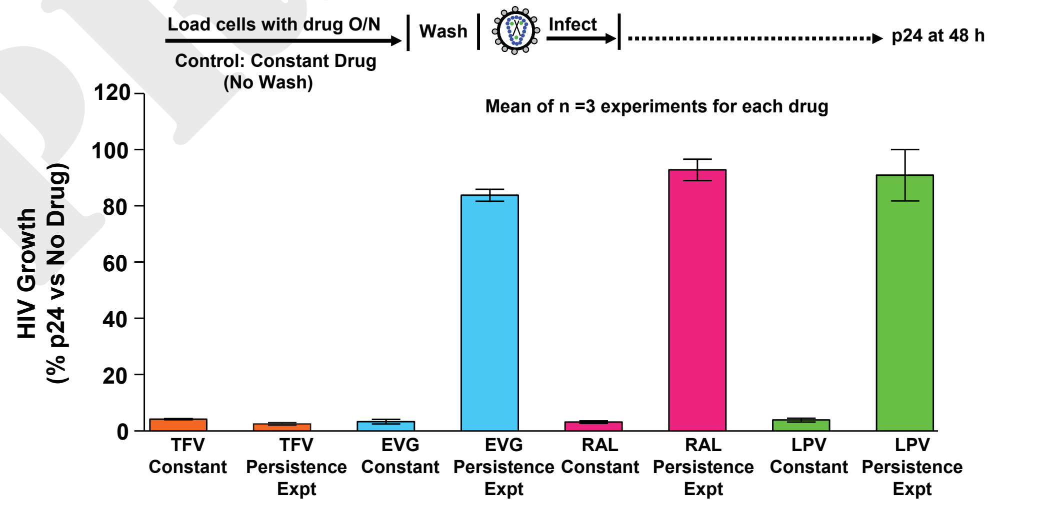
- TFV antiviral activity was sustained through 96 h following TFV removal, consistent with loading of cells with TFV-diphosphate that persists intracellularly and can mediate a post-antibiotic effect
- Neither EVG, RAL nor EFV prevented viral escape, indicating a lack of post-antibiotic effect

Figure 4. INIs Fail to Block Infection If Added Later Than 12-15 Hours Post-Infection



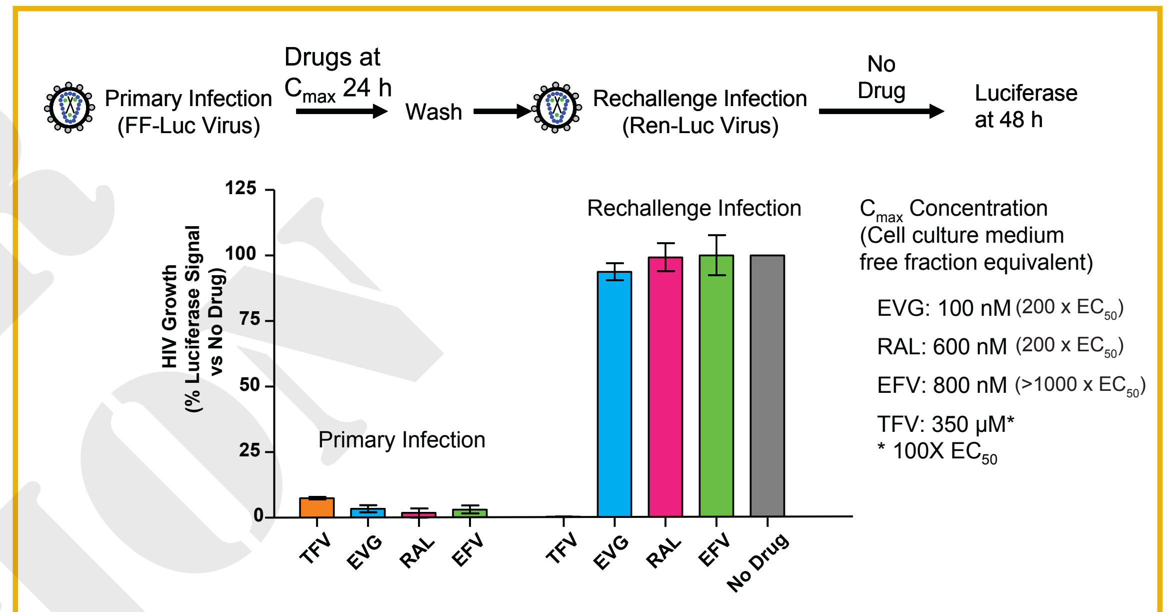
- HIV INIs, including EVG and RAL, inhibit the viral lifecycle up to 12-15 hours post-infection consistent with blockage of the integration step of the viral lifecycle

Figure 5. INIs Do Not Display Intracellular Antiviral Persistence Compared to TFV



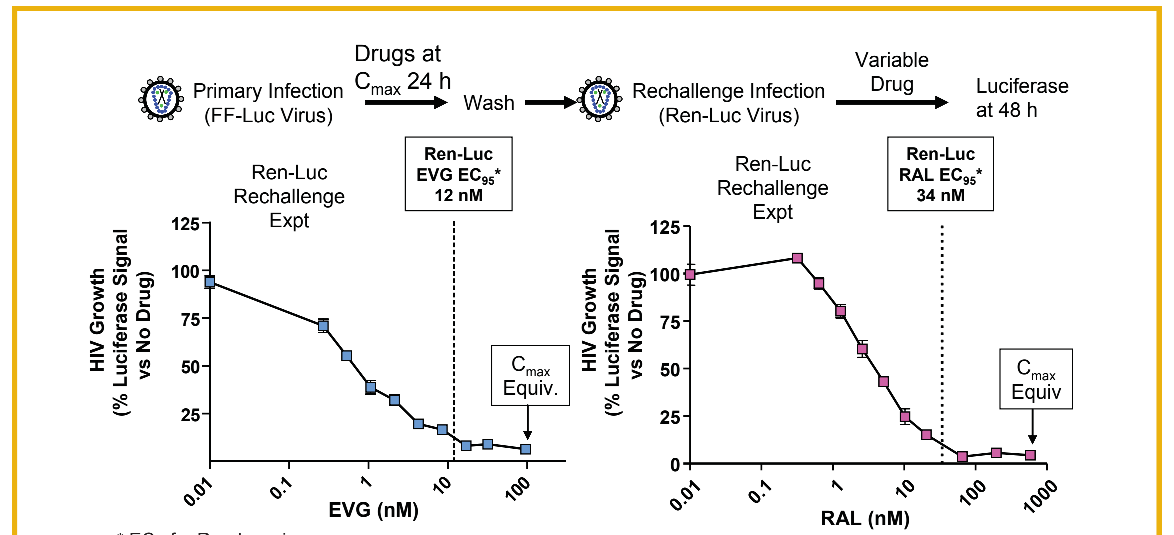
- EVG, RAL and LPV did not demonstrate antiviral activity when removed prior to infection
- Like PIs, the activity of INIs depends on their presence in the extracellular medium
- TFV demonstrated persistent antiviral activity, equivalent to constant drug exposure
- TFV could mediate a post-antibiotic effect due to intracellular persistence of TFV-diphosphate

Figure 6. INIs or EFV Do Not Protect Cells from Viral Rechallenge Following Drug Washout



- Primary infection was blocked by all ARVs at their respective C_{max} concentrations, or 100X EC_{50} for TFV
- Only TFV blocked subsequent rechallenge infection once drugs were removed
- INIs bound by IN during the primary infection could not protect cells against subsequent infections

Figure 7. Protection of Cells By INIs from Viral Rechallenge Only Occurs if INIs Are > EC_{95}



* EC_{95} for Ren-Luc virus

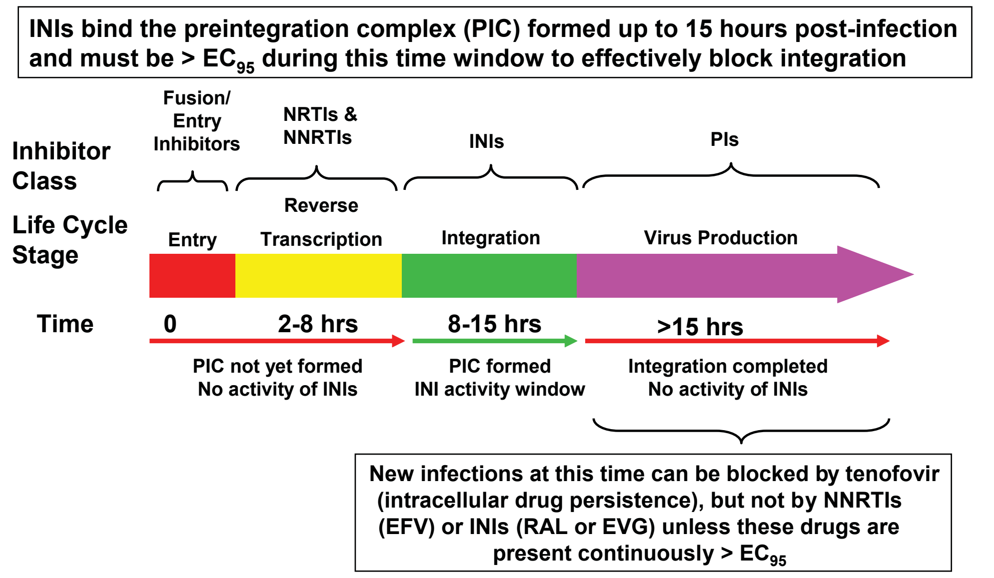
- C_{max} concentrations (based on free fraction) of both INIs strongly inhibited the initial infection
- Viral rechallenge of these cells at varying INI concentrations produced concentration-dependent infection
- INIs only protected cells against subsequent infections if they were > EC_{95}

Conclusions

- **Both EVG and RAL dissociated slowly from IN-DNA complexes with similar K_{off} rates**
- **No evidence of a post-antibiotic effect of HIV INIs was observed**
  - **The antiviral effects observed are consistent with the known modes of action of all the studied ARVs, including INIs**
  - **Of the ARVs studied, only tenofovir demonstrated activity consistent with a post-antibiotic effect**
- **INIs do not demonstrate antiviral intracellular persistence**
  - **Like PIs, the antiviral activity of INIs is dependent on their continuous presence in the extracellular environment**
- **Cells protected from initial viral challenge by INIs at C_{max} concentrations, can be reinfected by subsequent viral challenges when drug concentrations are < EC_{95}**

Discussion

Figure 8. ARVs, Including INIs, Must Be > EC_{95} During the Time of Their Mode of Action



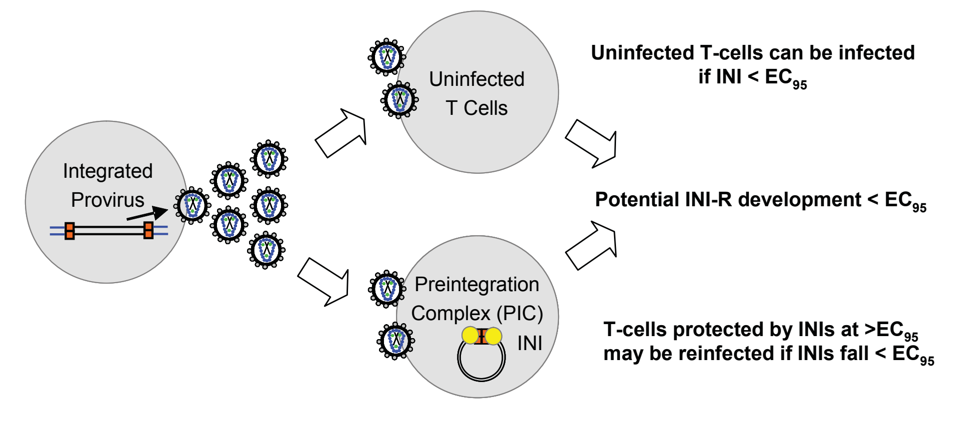
INIs bind the preintegration complex (PIC) formed up to 15 hours post-infection and must be > EC_{95} during this time window to effectively block integration

Inhibitor Class	Fusion/ Entry Inhibitors	NRTIs & NNRTIs	INIs	PIs
Life Cycle Stage	Entry	Reverse Transcription	Integration	Virus Production
Time	0	2-8 hrs	8-15 hrs	>15 hrs
	PIC not yet formed No activity of INIs		PIC formed INI activity window	Integration completed No activity of INIs

New infections at this time can be blocked by tenofovir (intracellular drug persistence), but not by NNRTIs (EFV) or INIs (RAL or EVG) unless these drugs are present continuously > EC_{95}

Figure 9. Model: Asynchronous Infections In HIV Infected Patients Necessitate Plasma INI Concentrations Be Maintained > EC_{95}

- In HIV patients, cells harboring proviral DNA established prior to INI therapy continually produce new virions, regardless of plasma INI concentration
- Unless blocked by an ARV, these virions form a source of new infections



Uninfected T-cells can be infected if INI < EC_{95}

Potential INI-R development < EC_{95}

T-cells protected by INIs at >EC_{95} may be reinfected if INIs fall < EC_{95}

- In bacterial post-antibiotic effect (PAE), the targets of antibiotics, e.g., ribosomal RNA, are always present in bacteria
  - Blockage of protein synthesis can result in bacterial cell death, even after plasma drug concentrations fall below the minimum inhibitory concentration, producing a post-antibiotic effect[1]
- In contrast, the preintegration complex (PIC), the target of HIV INIs, appears at a discrete stage of the viral lifecyle (Figure 8)
  - If the PIC forms when plasma INI concentration is < EC_{95}, integration may occur.
  - Established integrated proviruses in HIV-infected patients produce new virions continually which can infect T-cells asynchronously (Figure 9)
  - If INI plasma concentration is < EC_{95}, these new virions can escape INI inhibition and establish new infections unless blocked by other ARVs (Figure 9)
- Maintenance of ARV plasma drug levels > EC_{95} blocks infections and prevents emergence of drug resistance
- Identical principles appear to apply to integrase inhibitors whose activity is dependent on their continuous presence in the extracellular environment

References

1. Odenholt et al., 2001. Int. J. Antimicrobial Agents; 17:1-8
2. Xu et al., 2009. 49th ICAAC; Abstract H-934
3. Miller et al., 2008. 48th ICAAC/46th IDSA; Abstract H-898
4. Grobler et al., 2009. Methods; 47:249-253
5. Langley et al., 2008. Biochemistry; 47: 13481-13488