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**Comparative antiviral activity of integrase inhibitors in human
monocyte-derived macrophages and lymphocytes**

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Abstract

The activity of raltegravir and 4 other integrase inhibitors (MK-2048, L870,810, IN2 and IN5) was investigated in primary human macrophages, PBMC and C8166-lymphocytic T cells, in order to determine their relative potency and efficacy in different cellular systems of HIV infection. Raltegravir showed better protective efficacy in all cell types; MK-2048, L870,810 and IN5 showed a potent anti-HIV-1 activity in macrophages, while in lymphocytes only MK-2048 and L870,810 showed an inhibitory effect comparable to raltegravir. IN2 was a poorly effective anti-HIV-1 compound in all cellular systems. All effective integrase inhibitors exhibited a potent antiviral activity against both X4 and R5 HIV-1 strains. In general, raltegravir, MK-2048, L870,810 and IN5 showed anti HIV activity similar or slightly higher in macrophages compared to PBMC and C8166 T cells: for MK-2048, the EC_{50} was 0.4 nM, 0.9 nM, 11.5 nM in macrophages, in PBMCs and T cells, respectively; for L870,810, the EC_{50} was 1.5 nM, 14.3 nM and 10.6 nM, respectively; for IN5 the EC_{50} was 0.5 nM, 13.7 nM, and 5.7 nM, respectively.

1. Introduction

Despite the extensive research and successful discovery of antiviral drugs starting over 25 years ago, from the HIV identification, the eradication of HIV-1 infection and cure from AIDS still remain a long term challenge (Dieffenbach and Fauci, 2011; Richman et al., 2009)

The development of new powerful drugs acting at different stages of HIV-1 infection represents a priority, particularly for cells, such as monocyte-macrophages (M/M), whose infection is persistent and productive for long periods of time. Understanding the anti-HIV-1 activity of antiretroviral drugs in M/M is a crucial point in designing therapeutic strategies aimed at achieving an optimal therapeutic effect in all tissue compartments where the virus hides and replicates, such as the central nervous system (CNS). Indeed, M/M, together with memory CD4⁺ T cells, represent the most important viral reservoirs for HIV-1, in which the virus can persist and survive for very long time in the body (Bagnarelli et al., 1996; Cassol et al., 2006; Chomont et al., 2009; Chomont et al., 2011; Chun et al., 1995, 2003; Finzi et al., 1997; Kogan and Rappaport, 2011; Pierson et al., 2000; Venzke and Keppler, 2006). Chomont et al. recently showed that HIV-1 persists in a transcriptionally silent form in central memory and transitional memory CD4⁺ T-lymphocytes, which are capable of low-level proliferation. These latently infected cells could expand the viral reservoir continuously and render the infection impossible to eradicate from the body in the absence of specific pharmacological interventions (Chomont et al., 2011; Dieffenbach and Fauci, 2011; Pomerantz, 2001; Pomerantz and Horn, 2003). Infected macrophages, which can survive and produce virus for long periods, play an important role in all phases of HIV-1 infection and in various aspects of the disease process, acting as a vehicle for virus dissemination, and damage to bystander cells and therefore representing another major and important obstacle to the complete eradication of virus even in the presence of antiretroviral therapy (Alexaki et al., 2008; Aquaro et al., 1997, 1998, 2002a,b; Aquaro and Perno, 2005; Coleman and Wu, 2009; Herbein et al., 2010; McGrath, 1996; Sharova et al., 2005).

The peculiar characteristics of HIV-1 replication in M/M, is quite different from that observed in lymphocytes, and makes possible that HIV-1 inhibitors could show different activity and effects in these two cell types (Garaci et al., 2003; Kaul et al., 2001); Aquaro and colleagues have already shown that nucleoside analogues (AZT, ddI, ddC, d4T, PMEA, 3TC etc.) have greater activity in M/M than in lymphocytes (Aquaro et al., 1997). Due to the different cellular metabolism and different replicative capacity of HIV-1 in M/M and lymphocytes, and because many drugs are known to act differently in these two cell types, it is conceivable that the efficacy of integrase Inhibitors (INIs) in M/M and lymphocytes may be different.

INIs target the key enzyme integrase of HIV-1, essential for viral replication, and thus they are some of the most promising anti HIV-1 compounds (Ceccherini-Silberstein et al., 2009; McNeely et al., 2008; Schafer and Squires, 2009). For instance, one of the most exciting recent advances in HIV-1 pharmacotherapy has been the approval in the October 2007 by Food and Drug Administration (FDA) of the first INI, the new pyrimidone carboxamide raltegravir (RAL) provided with a high potency and generally well tolerability (Cocohoba and Dong, 2008; Goffinet et al., 2009; Moltò et al., 2011; Steigbigel et al., 2008; Summa et al., 2008).

Consistent findings show an unusual drop of viral load in the first weeks of RAL treatment, with an almost absent second phase decay (Hicks and Gulik, 2009; Markowitz et al., 2006; Murray et al., 2007). The reason of this effect has not yet been elucidated, and a possibility of a peculiar effect of RAL on HIV-1 reservoirs, including M/M could be postulated. The knowledge about the efficacy of INIs on M/M, that is actually very limited, represents a crucial task for improving therapeutic approaches against HIV infection.

For all these reasons, the aim of the present study was to investigate in detail the anti-HIV-1 activity of RAL and of other INIs (MK-2048, L-870,810, IN2 and IN5) (Hazuda et al., 2000; Little et al., 2005; Serrao et al., 2009; Vacca et al., 2007; Wai et al., 2007) in M/M, PBMCs and lymphocytes, in order to determine their relative potency and efficacy in different cellular systems of HIV infection.

2. Materials and Methods

2.1 Cells

Human T-lymphocytic C8166 T cells were obtained from the American Type Culture Collection (Manassas, VA).

Human primary M/M were prepared and purified as described in published procedures (Bagnarelli et al., 1996; Cenci et al., 1997; Perno et al., 1998). Briefly, M/M were obtained from the blood of healthy HIV-seronegative donors. First peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-Hypaque gradient centrifugation and seeded in plastic 48-well plates (Costar, Cambridge, MA) at a density of 1.8×10^6 cells/ml in RPMI 1640 (Gibco, Gaithersburg, MD) supplemented with 50 U/ml penicillin, 50 μ g/ml streptomycin, 2 mM L-glutamine and 20% heat-inactivated, mycoplasma- and endotoxin- free fetal calf serum (HyClone, Logan, UT) (complete medium). Cell cultures were incubated in a humidified atmosphere with 5% CO₂ at 37°C. Non-adherent cells were removed 6 days after seeding by repeated gentle washing with warmed RPMI 1640, leaving a monolayer of adherent cells, which were incubated in complete medium as previously described. Adherent cells obtained using this technique generally consisted of >95% differentiated M/M (at cytofluorimetric analysis, more than 95% of cells were CD14⁺ CD4⁺ CD3⁻). PBMCs were obtained by Ficoll-Hypaque gradient centrifugation and seeded in T25 flask (Costar, Cambridge, MA) at a density of 1.0×10^6 cells/ml in RPMI 1640 (Gibco, Gaithersburg, MD) supplemented with 50 U/ml penicillin, 50 μ g/ml streptomycin, 2 mM L-glutamine and 20% heat-inactivated, mycoplasma- and endotoxin- free fetal calf serum (HyClone, Logan, UT) (complete medium) supplemented with 2 μ g/ml phytohemagglutinin (PHA) and with 50 U/ml recombinant interleukin-2 (IL-2). PBMCs and M/M used in the present study were from different blood donors.

2.2 Viruses

Two different HIV-1 isolates were used in this study. A monocytotropic HIV-1 R5 isolate, such as HIV-1 Bal, was used in all experiments involving primary M/M and PBMC. Characteristics and genomic sequence of this strain have been previously described (Cenci et al., 1997). The virus was expanded in M/M, whose supernatants were collected, filtered (0.22 μ M) and stored at -80°C before use. The virus obtained after previous expansion was sequenced (at least portions of *pol* and *env*), and found very stable over few passages. The M/M and PBMC infections were performed using the same viral stock.

The prototypic HIV-1 lymphocytotropic X4 strain, namely HIV-1III_B, was used to infect C8166 T cells. Cell free virus stock was ultracentrifuged, filtered (0.22 μ M) and stored at -80°C.

2.3 Compounds

RAL, synthesized under GMP conditions, was obtained from Merck; a stock solution, diluted in PBS, was made fresh for each experiment. The compounds MK-2048, L-870,810, IN2, IN5 were synthesized at Merck, diluted in PBS and stored at -20°C before use. Their structures are presented in Fig.1. The NRTI inhibitor 3'-azido-2', 3'-dideoxythymidine (AZT), was used as control at a concentration known to be active against HIV-1 replication (1 μ M).

2.4 HIV-1 infection

C8166 T cells were exposed to several doses of INIs (1000 nM, 250 nM, 60 nM, 15 nM, 4 nM, 1 nM, 0.25 nM) for 20 min, and then challenged with the lymphocytotropic X4 III_B (3,000 pg/ml of HIV-1 p24 gag Ab). After 2h of incubation at 37°C in a humidified atmosphere supplemented with 5% CO₂, C8166 T cells were extensively washed with warm RPMI 1640 to remove the excess virus, and then complete medium containing the same concentration of the INIs was added, when appropriate. The antiviral activity of INIs in infected C8166 T cells was assessed by analysis of cytopathic effect at days 5 and 7 after HIV infection. The EC₅₀ and EC₉₀ were calculated on the

basis of the effective drug concentrations, where 50% and 90% of syncytium formation was inhibited (representing the EC₅₀ and EC₉₀), respectively.

Syncytia were evaluated by two independent observers using a semi quantitative score system based on the following criteria: (0) no syncytia; cells with few multinucleated giant cells (10-20% of the fields) were scored as 1+; wells with syncytia in 20-50% of the fields were scored as 2+; cells with syncytia in 50-90% of the wells were scored as 3+; wells with syncytia in all fields and evidence of cytoplasmic ballooning were scored as 4+.

The activity of INIs was also tested in primary M/M and PBMCs. Based on the EC₅₀ calculated in C8166 T Cells, two doses higher and two doses lower of EC₅₀ for each INI have been used. M/M and PBMC were pre-treated 20 min with INIs, infected with 3,000 pg/ml of R5 Bal and incubated at 37°C in a humidified atmosphere supplemented with 5% CO₂.

Supernatants of infected PBMCs (collected at 6 days) and of infected M/M (collected at 14 and 21 days) were assessed to determine virus production in the presence or absence of INIs, by measurement of HIV p24 gag production using a commercially available HIV antigen ELISA kit (Bio-Rad, France). The experiments were performed at least twice in triplicate.

The geometric mean of p24 gag production of replicates in each experiment was used to determine the EC₅₀ and EC₉₀, by linear regression of the log of the percent HIV-1-p24 production (compared to untreated controls) versus the log of the drug concentration.

2.5 Drug toxicity

Drug toxicity was assessed in the absence of viral infection. Uninfected C8166 T cells, M/M and PBMCs were treated in the presence of different concentrations of INIs (up to 200 µM). Cell viability was visually assessed, and compared to untreated controls, using the trypan blue exclusion method. In the case of M/M, cells were gently detached from the wells as described elsewhere (Cenci et al., 1997). Briefly, cells were exposed to dye, and then visually examined to determine whether cells take up or exclude the dye. The live cells that possess intact cell membranes exclude

trypan blue, whereas dead cells do not. The selectivity index (SI) was calculated based on the ratio of the 90 % cytotoxic dose (TC_{90}) to the 90 % antiviral effective dose (EC_{90}).

2.6 Flow cytometric analysis of cellular apoptosis

PBMCs (100×10^3 cells/well in 1ml complete RPMI-1640) were added to: mock HIV-1 infected M/M, HIV-1-infected M/M, and HIV-1-infected M/M INIs-exposed (after 21 days of HIV-1 macrophage infection; at EC_{90} of different drugs). After 5 days, cellular apoptosis in PBMC was evaluated by flow cytometric analysis as reported by Pollicita et al. (2009). Briefly, after 5 days of M/M and PBMC co-culture, PBMCs were collected and centrifuged at 1,600 rpm for 10 min. Pellets were washed with PBS, placed in ice, and permeated with ice-cold 70% ethanol for 30 min. The aliquots were centrifuged at 1,500 rpm for 10 min, the pellets were washed with PBS, incubated with propidium iodide (PI; 100 μ g/ml, SIGMA-Aldrich, Germany) and RNase (250 μ g/ml Qiagen, Mi, Italy) at 4°C for 2h in the dark. Samples were then washed twice with PBS and PI-stained cells were analyzed by monitoring the incorporation of intracellular PI with a FACScan flow cytometer. A total of 10^5 events were collected for each sample. Data were acquired and analysed by the Lysis II program (Becton Dickinson, Buccinasco, Italy).

3. Results

3.1 Antiviral activity of INIs in C8166 T cells

Viral replication in HIV-1-infected C8166 T cells treated with several doses of INIs, was assessed at 5 and 7 days after infection by a cytopathic effect based on the analysis of syncytium formation and cellular aggregation. Five days after HIV-1 infection, the cytopathic effect was drastically reduced in a dose-dependent manner in the presence of all INIs, with the exception of IN2, which showed poorly antiviral activity at all tested concentrations. The EC_{50} and EC_{90} of the INIs are reported in Table 1. The EC_{50} of all compounds was in the same range of activity, between 5.0 and 11.5 nM, with the exception of IN2 for which the EC_{50} was >200 nM (data not shown). EC_{90} was the lowest for RAL (15.6 nM) versus the highest 57.2 nM for MK2048, (IN2 did not reach the EC_{90} at any concentration tested) (Table 1). Same results were obtained at 7 days after infection (data not shown).

3.2 Antiviral activity of INIs in Macrophages

The effectiveness of all compounds was also assessed in primary human M/M. A significant dose-dependent antiviral activity of different INIs was achieved in M/M at 14 days after HIV-1 infection (Figure 2A).

The 6.9 nM dose of RAL led to a reduction of p24 gag Ag production down to about 90%, while the 0.3 nM dose of RAL was sufficient to inhibit 50% of virus production in infected M/M (Table 1). Similar results were obtained with MK-2048 and IN5; they showed strong antiviral activity against HIV-1 Bal, with an EC_{50} of 0.4 nM for MK-2048 and an EC_{50} of 0.5 nM for IN5 (Table 1). Some differences, not statistically significant, among these two INIs and RAL were found in the EC_{90} , where the EC_{90} for RAL was lower compared to the EC_{50} for the other INIs (RAL: 6.9 nM, MK-2048: 43.4 nM, and IN5: 21.3 nM) (Table 1).

The compound IN2, also in HIV-infected M/M, did not reach the EC_{50} or EC_{90} values.

3.3 Antiviral activity of INIs in PBMCs

Fresh primary human PBMCs were used to confirm the antiviral activity of INIs. Viral replication of HIV-1 Bal in PBMCs after 6 days of infection with different doses of INIs was investigated (Figure 2B).

RAL showed a stable antiviral activity in acutely infected PBMCs until the end of the experiment, with an EC₅₀ of 1.0 nM and an EC₉₀ of 14.5 nM (Table 1), slightly higher than those observed in M/M. MK-2048 showed an inhibitory effect (0.9 nM) comparable to that observed with RAL (1.0 nM). However, also in these cells, the EC₉₀ for MK-2048 (57.5 nM) was higher compared to RAL (14.5 nM) (Table 1). L870,810 and IN5 were active, but showed an higher EC₅₀ (range 14.3 nM-13.7 nM) and EC₉₀ (range >120nm-39.6) compared to that observed with RAL. Also in PBMC, the IN2 compound did not reach EC₉₀ at any concentration tested (Figure 2B).

Interestingly, all effective INIs, showed slightly higher values of EC₅₀ and EC₉₀ in PBMCs compared to M/M. However, all these differences were not statistically significant.

3.4 Drug toxicity

Treatment of M/M, C8166 T Cells and PBMCs with concentrations of INIs up to 200µM (markedly higher than the antiviral effective concentrations) showed no decrease in cell number, thus suggesting the absence of major toxicity. Only L870,810 showed a partial cellular toxicity at the highest concentration (200 µM). In fact, all these compounds showed a selective index (SI) >1000 (with the only exception of L870,810 in PBMC), as shown in Table 1.

Thus, the antiviral activity observed in our experiments can be attributed only to the inhibitory effects of the INIs and not to an alteration of cellular metabolism or cellular death.

3.5 Effects of INIs on preventing apoptosis in PBMCs co-cultured with HIV-1-infected M/M

Apoptosis has been evaluated and statistically analyzed in PBMCs co-cultured with HIV-1-infected M/M, in PBMCs co-cultured with HIV-1-infected M/M INIs treated with INIs and, and in PBMCs co-cultured with mock HIV-infected M/M and not treated with INIs (negative control). A significant induction of PBMC apoptosis was observed after 5 days of co-culture with HIV-1-infected M/M (29% of apoptotic cells vs 9% in the mock control, $p < 0.001$). All INIs, with (again) the only exception being IN2, strongly reduced the apoptosis in PBMC co-cultured with R5 HIV-1-infected M/M ($p < 0.001$) (Figure 3).

4. Discussion

Our experiments were focused to investigate the antiviral activity of RAL and other INIs in M/M, PBMCs and a T lymphocytic-cell line.

RAL is a new drug belonging to a new class of antiretrovirals (INIs), which has demonstrated an exquisite potency, a clean safety profile also at once-daily dosing of 800 mg/day, and to not accumulate in PBMCs, with intracellular concentrations being about 1/10 of the concentrations in plasma (Goffinet et al., 2009; Moltó et al., 2011; Murray et al., 2007; Steigbigel et al., 2008; Summa et al., 2008).

In our study, all INIs tested showed potent antiviral activity against both R5 and X4 HIV-1 strains, with IN2 as the only exception, which was poorly effective in all our cellular systems. The doses of RAL and other INIs able to inhibit the HIV-1 in M/M were lower compared to those for PBMCs and T cells (Table 1), although these differences were without statistical significance. This biological effect could be correlated to the peculiar characteristics of HIV-1 replication in M/M, which is quite different from that observed in CD4⁺ T lymphocytes (Aquaro et al., 2002a,b; Badley et al., 1997; Bagnarelli et al., 1996; Garaci et al., 1999; Gendelman et al., 1986; Michelini et al., 2010; Orenstein et al., 1988). Indeed, M/M are resting, terminally differentiated cells that undergo

replication only under very peculiar conditions and situations; this makes the whole integration phenomenon more difficult, as it occurs during the replication cycle of cells (Kelly et al., 2008). Therefore, it is conceivable that HIV integration, occurring slower in M/M due to the lower cycle metabolism, should be more easily perturbed by concentrations of INIs even lower than those effective in replicating cells (Kelly et al., 2008).

This event may have important implications in the pathogenesis of HIV-1 disease, because of its impact on resting/persistently-infected HIV-1 reservoirs, and on the transmission of virus to uninfected T-lymphocytes. This may result in a perturbed infection of T-lymphocytes and subsequent altered apoptosis. In fact, it is already known that M/M can recruit lymphocytes and trigger their death, through several events, mediated by viral proteins (such as nef and gp120), or by chemokines and other factors produced during HIV-1 infection (Badley et al., 1997; Charpentier et al., 2008).

In this respect, it is crucial to know whether RAL and other INIs are able in preventing or reducing viral transmission from M/M to lymphocytes. In the present study, we showed that RAL is able to remarkably reduce the apoptosis in PBMCs co-cultured with R5 HIV-1-infected M/M (Figure 3). Notably, M/M are the key (and almost unique) target of HIV in the brain, where the infection causes a typical HIV encephalopathy. It has been demonstrated that infected M/M are highly efficient to enter in CNS by crossing the blood brain barrier (BBB); once M/M are in the CNS, they continue to produce various substances and viral proteins that can alter the BBB, activate other cells and damage neurons and astrocytes (Herbein et al., 1998).

The good penetration of RAL, recently demonstrated in a number of publications, and the remarkable activity of RAL in M/M, suggest that RAL, and perhaps other INIs, represent a therapy of choice to prevent and control the replication of HIV in the brain, particularly in patients with signs and symptoms of HIV-associated damage of the central nervous system (Croteau et al., 2010; Yadav and Collman, 2009).

In conclusion, HIV replication is inhibited by INIs in M/M at similar, or even lower, concentrations than those active in PBMCs. This supports the hypothesis that RAL (and other INIs) are able to control the spreading of HIV in M/M and in actively replicating T cells. Thus, they represent one of the most promising classes of compounds in current development as well as in clinical practice.

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Conflict of interest F. Ceccherini-Silberstein has received funds for attending symposia, speaking and organizing educational activities from Abbott, Merck Sharp & Dohme, Janssen Cilag, and Virco.

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The other authors declare no competing interests.

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Legends to figure

FIGURE 1: Chemical structures of the INIs tested

FIGURE 2: Antiviral activity of INIs in M/M and PBMCs.

M/M (Panel A) and PBMCs (Panel B) were infected with 3000 pg/ml of HIV-1 Bal, in the presence of RAL, or MK2048, or L870,810, or IN5, or IN2, at indicated doses.

AZT was used as antiviral control, at a concentration known to be active against HIV-1 replication (1 μ M).

Supernatants collected at the 14th day for M/M and at the 6th day for PBMCs were assessed to HIV-1 p24 Ag Elisa test. A representative experiment performed in triplicate is shown. The results are presented as mean values with standard deviations.

FIGURE 3: Apoptosis reduction in PBMC cocultured with HIV-1-infected M/M by the presence of INIs.

Apoptosis has been evaluated in PBMCs co-cultured with: mock HIV-1 infected M/M (negative control), HIV-1 infected M/M and with HIV-1-infected M/M INIs treated (at EC₉₀ of different compounds).

The apoptotic cells were stained with propidium iodide and analysed by flow cytometry. A significant induction of PBMC apoptosis was observed after 5 days of co-culture with HIV-1-infected M/M (29% of apoptotic cells, vs 9% in the mock control, $p < 0.001$).

The bars represent the percentage of apoptosis in the presence or absence of INIs and AZT. The percentage of apoptotic PBMCs co-cultured with mock HIV-1-infected M/M has been subtracted from all samples. Statistical analysis was performed by χ^2 test. * = $P < 0.001$.

	C8166			M/M			PBMC		
	EC ₅₀ ^a nM	EC ₉₀ ^b nM	SI [*]	EC ₅₀ ^c nM	EC ₉₀ ^d nM	SI [*]	EC ₅₀ ^e nM	EC ₉₀ ^f nM	SI [*]
RAL	5.0 ±2.4	15.6 ±2.6	>10.000	0.3 ± 0.1	6.9 ±0.1	>20.000	1.0± 0.2	14.5 ±2.0	>10.000
MK2048	11.5±5.4	57.2 ±6.0	4651	0.4 ±0.1	43.4±17.8	6500	0.9 ±0.1	57.5 ±4.9	3700
L870,810	10.6 ±1.4	48.4 ±0.1	2600	1.5 ±0.2	102.5 ±13.3	1075	14.3±0.2	>120	>500
IN5	5.7±2.7	15.4±0.6	>10.000	0.5±0.1	21.3±5.6	7900	13.7±1.7	39.6±0.5	5100

Table 1: Comparative anti-HIV efficacy of INIs in HIV-1 (III_B)-infected C8166 T cells and HIV-1 (BaL- infected M/M and PBMCs.

a 50% Effective concentration, or compound concentration required to inhibit HIV-1(III_B) syncytium formation in virus-infected C8166 T cells by 50% at day 5 post infection.

b 90% Effective concentration, or compound concentration required to inhibit HIV-1(III_B) syncytium formation in virus-infected C8166 T cells by 90% at day 5 post infection.

c 50% Effective concentration, or compound concentration required to inhibit HIV-1(BaL) p24 production in virus-infected M/M by 50% at day 14 post infection.

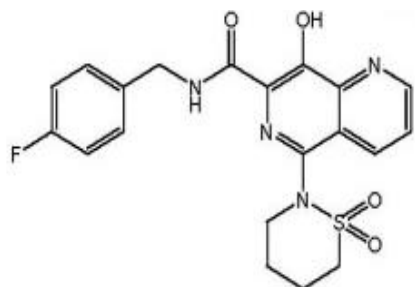
d 90% Effective concentration, or compound concentration required to inhibit HIV-1(BaL) p24 production in virus-infected M/M by 90% at day 14 post infection.

e 50% Effective concentration, or compound concentration required to inhibit HIV-1(BaL) p24 production in virus-infected PBMC by 50% at day 6 post infection.

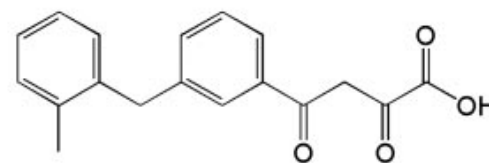
f 90% Effective concentration, or compound concentration required to inhibit HIV-1(BaL) p24 production in virus-infected PBMC by 90% at day 6 post infection.

* Selective index. ratio TC₉₀/EC₉₀.

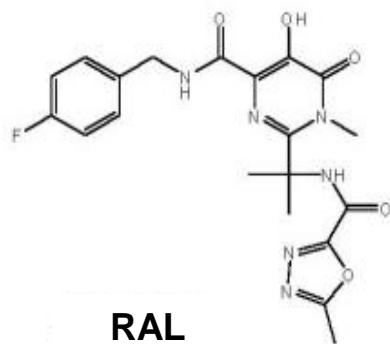
FIGURE 1



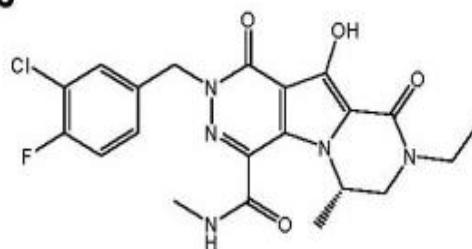
L870,810



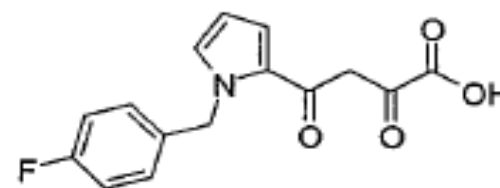
IN2



RAL



MK-2048



IN5

FIGURE 2A

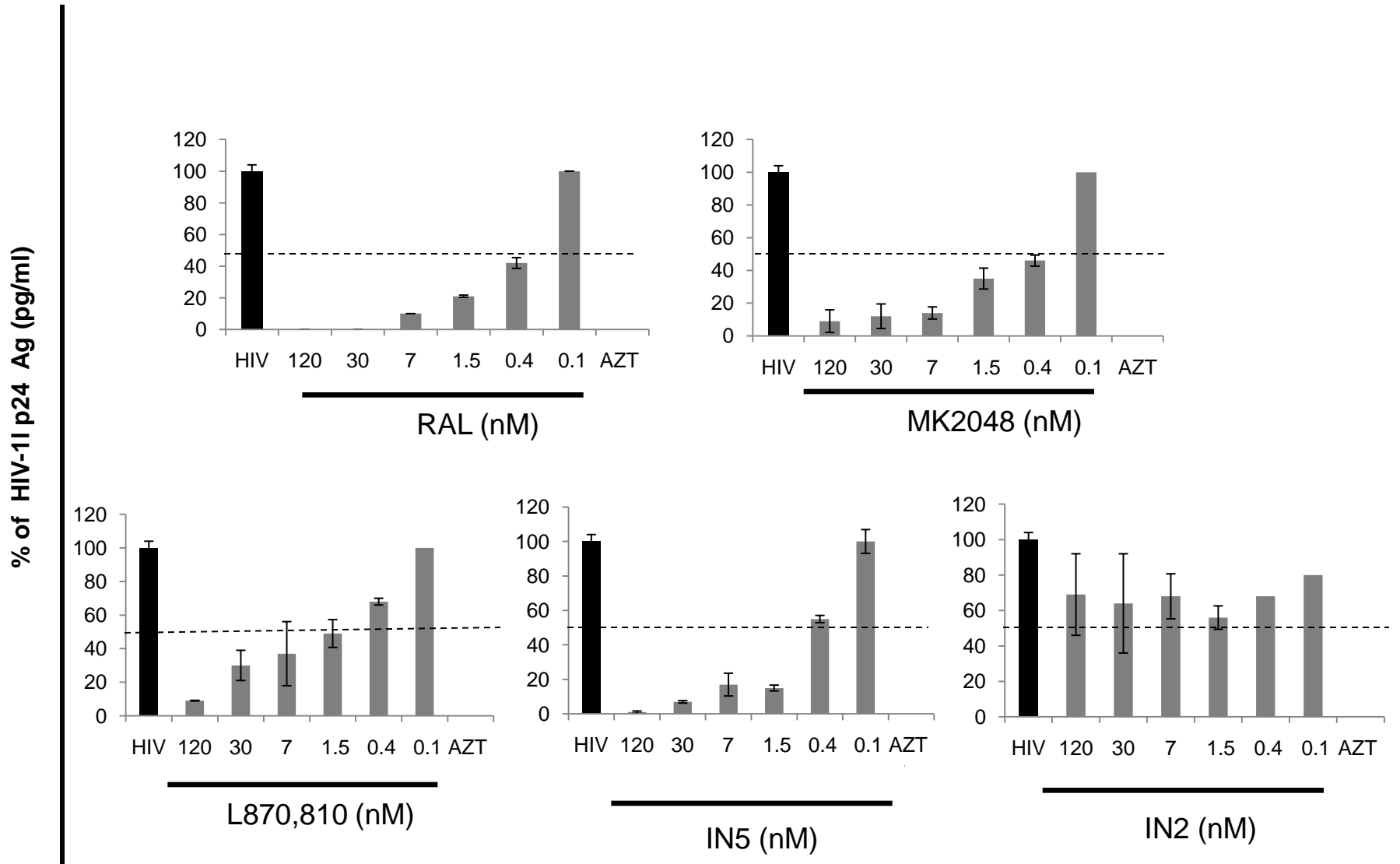


FIGURE 2B

% of HIV-1 p24 Ag (pg/ml)

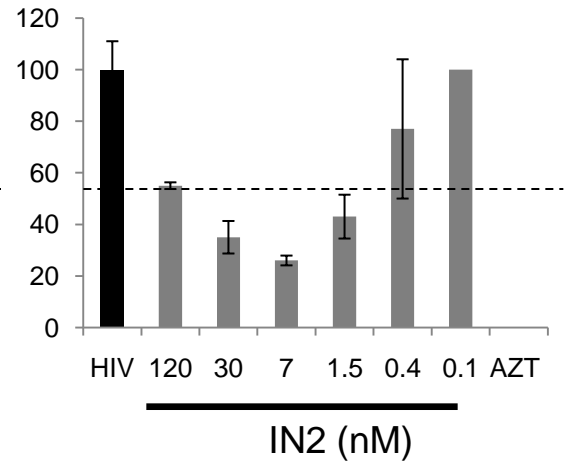
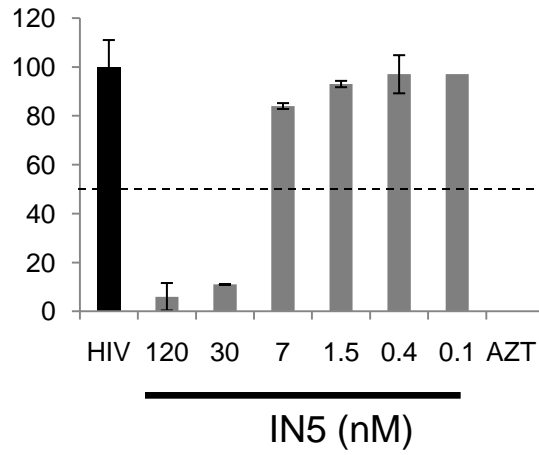
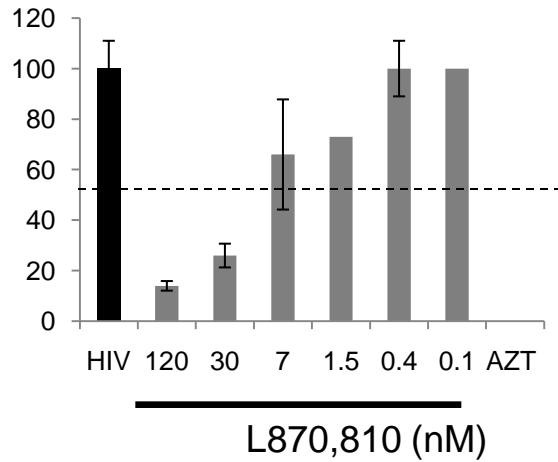
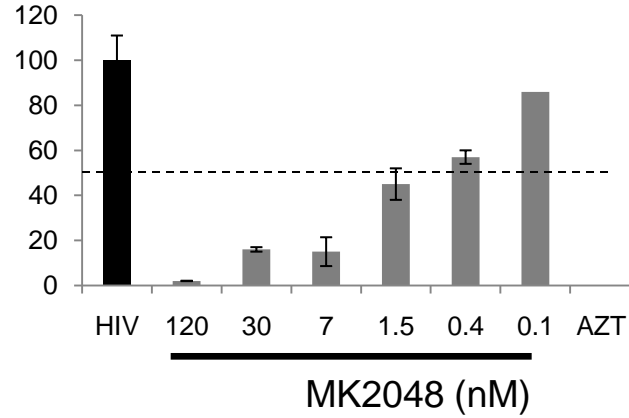
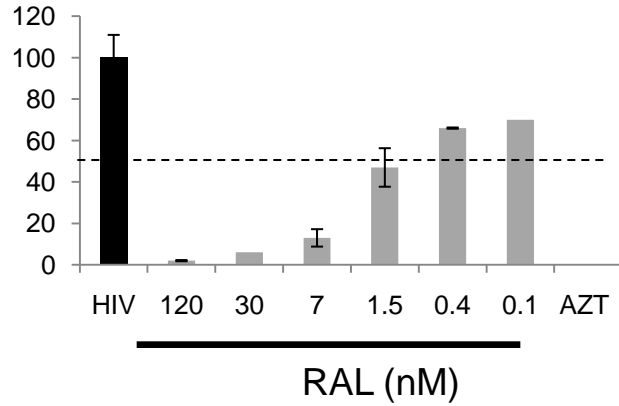


FIGURE 3

