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In vitro evaluation of the effect of temporary removal of HIV drug pressure

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Abstract

We tried to establish whether MT-4 cells that were infected with HIV-1(HTLV-III_B) at a high multiplicity of infection (m.o.i. = 1), and subsequently treated with high concentrations of anti-HIV drugs for several days, would be able to resume virus production after the antivirals are washed away. The HIV inhibitors studied were the nucleoside reverse transcriptase inhibitors (NRTIs) zidovudine and lamivudine, the non-nucleoside reverse transcriptase inhibitors (NNRTIs) nevirapine, delavirdine and loviride, the acyclic nucleoside phosphonate RT inhibitor (*R*)-9-(2-phosphonylmethoxypropyl)adenine (tenofovir) and the protease inhibitors (PIs) saquinavir, indinavir and ritonavir. The compounds, at 50 and 500 times their 50% inhibitory concentration (IC_{50}, determined at a m.o.i. = 0.01), were added immediately after virus adsorption and removed after an incubation period of 0 (wash control), 24, 48 or 72 h. Virus breakthrough was monitored by microscopical examination of cytopathicity, viral infectivity (yield) and p24 levels in the supernatant. The presence of HIV-1(HTLV-III_B) proviral DNA was determined after a 72-h incubation period. None of the antiviral drugs studied was able to prevent resumption of viral growth after removal of the compound. Tenofovir, lamivudine and the NNRTIs nevirapine, delavirdine and loviride, at 500 times their respective IC_{50}, were able to delay viral breakthrough for approximately 2–3 days. The NRTI zidovudine and the PIs saquinavir, indinavir and ritonavir, under the same conditions, were not able to delay viral breakthrough at all. Virus recovered upon treatment proved as sensitive to the anti-HIV drugs as wild-type virus. Our results suggest that viral replication at the cellular level was not completely inhibited by drug monotherapy. Consequently, virus rebounded when drug therapy stopped. In conclusion, our findings suggest that drug holidays would result in viral breakthrough, even after virus replication has been previously suppressed by adequate drug levels.

Keywords: Antiretroviral therapy; Anti-HIV drug; Reverse transcriptase; Protease; Breakthrough; Compliance

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1. Introduction

During the last decade intensive efforts have been undertaken to develop drugs against human immunodeficiency virus (HIV), the causative agent of AIDS. At present, 14 compounds have been approved by the US Food and Drug Administration for the treatment of HIV infections: the dideoxynucleoside analogs zidovudine (AZT), didanosine (ddI), zalcitabine (ddC), stavudine (d4T), lamivudine (3TC) and abacavir (ABC), the NNRTIs nevirapine (BI-RG587), delavirdine (U-90152), efavirenz (DMP266) and the protease inhibitors saquinavir (Ro31-8959), ritonavir (ABT-538), indinavir (MK-639), nelfinavir (AG-1343) and amprenavir (VX-478). Drug monotherapy has proven insufficient to provide long-term suppression of HIV-1 replication in HIV-1-infected individuals. Therefore, combination therapies with two or more drugs are required for effective treatment of AIDS (Caliendo and Hirsch, 1994; Balzarini et al., 1995; De Clercq, 1996; Johnson, 1996; Balzarini, 1999). However, more than 40% of Americans with HIV do not take their drugs as prescribed. Doctors and patients complain that the current regime of anti-HIV drugs is hard to comply with. Patients have to take sometimes dozens of pills a day, at set times, some with food, some without. Patients are giving themselves drug holidays, taking themselves off the drugs for anywhere between a few days to several weeks. The average drug holiday has been estimated as 2 weeks.

In this work, we wanted to study what happens with viral replication at the cellular level after cessation of drug monotherapy, as in the case of the lack of compliance of the patient.

2. Materials and methods

2.1. Compounds

AZT (zidovudine) and α-APA R89439 (loviride) were synthesized as previously described (Horwitz et al., 1964; Pauwels et al., 1993). 3TC (lamivudine) was a gift from Glaxo Wellcome, UK U-90152. (Delavirdine) was kindly provided by Pharmacia & Upjohn (Kalamazoo, MI) by B. Bruce. BI-RG587 (nevirapine) was obtained from Boehringer Ingelheim (Ridgefield, CN). (*R*)-9-(2-phosphonylmethoxypropyl)adenine PMPA (tenofovir) was kindly provided by Gilead Sciences (Foster City, CA). Saquinavir (Ro31-8959) was a gift from Dr N. Roberts (Roche Products Limited, Welwyn Garden City, UK). Indinavir (MK-639, formerly L735-524) was obtained from Dr J.R. Huff (Merck Research laboratories, West Point, PA) and ritonavir (ABT-538) was kindly provided by Abbott Laboratories (Abbott Park, IL).

2.2. Cells and viruses

MT-4 cells (Miyoshi et al., 1982) were used in all the experiments. The cells were grown and maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 0.1% sodium bicarbonate and 20 μg/ml gentamicin (culture medium). HIV-1(HTLV-III_B) was used in all the experiments (Popovic et al., 1984). The virus strain was propagated in MT-4 cells. Titer of virus stock was determined in MT-4 cells, and the virus stock was stored at -80°C until use.

2.3. Antiviral assay

Inhibitory effects of the compounds on HIV-1 replication were monitored by the inhibition of virus-induced cytopathic effect (CPE) in MT-4 cells as previously described (Pauwels et al., 1988). Briefly, HIV-1(HTLV-III_B) was added to a flat-bottomed microtiter tray containing various concentrations of the test compounds. MT-4 cells were added at a final concentration of 1.5×10^5 cells/ml resulting in a m.o.i. (multiplicity of infection) = 0.01. After a 5-day incubation at 37°C, the number of viable cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Cytotoxicity of the compounds for mock-infected MT-4 cells was also assessed by the MTT method.

2.4. Long-term culture of HIV-1-infected cells

MT-4 cells were infected with HIV-1(HTLV-III_B) at a m.o.i. = 1. FACS analysis revealed that at a calculated m.o.i. = 1, approximately 60% of the cells became infected in the first round of replication. After a 1 h virus adsorption period, cells were washed twice with culture medium to remove unadsorbed viral particles. Infected cells were suspended at 3×10^5 cells/ml and immediately thereafter 1 ml of the cell suspension was brought into centrifugation tubes containing various concentrations of the test compounds (1 ml volumes). Compounds were removed by 3 centrifugation steps after a treatment period of 0 (wash control), 24, 48 or 72 h in 24-well microtiter trays. Virus breakthrough was monitored by microscopical examination of cytopathicity. Culture supernatants were collected and examined for viral infectivity and for their p24 antigen level by an antigen capture ELISA kit (NEN Life Science Products, Zaventem, Belgium). Culture supernatants were also used to make virus stocks. Sensitivity of these virus stocks towards the antiviral drugs was assessed as described above.

Appearance of HIV-1(HTLV-III_B) proviral DNA was determined using 1.5×10^4 cells infected with HIV-1(HTLV-III_B) at a m.o.i. = 1 and treated with test compounds for 72 h. The cells were transferred to a 0.5 ml Eppendorf tube, overlaid with 20 μl of mineral oil and lysed at 94°C for 20 min. PCR was performed on a PE-9600 instrument in a total volume of 50 μl with 10 mM Tris–HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each deoxynucleoside triphosphate, 1 μM of each primer and 1.25 U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus). Two primer pairs (synthesized by A. Van Aerschot, Rega Institute for Medical Research, Leuven, Belgium) were used: the repliprimers to amplify a 263 bp fragment in the *pol* region of HIV-1 and two globin primers (primers PCO3 and KM38) to amplify a 167-bp fragment of human β-globulin (Vandamme et al., 1995). A total of 8 μl of each sample was electrophoresed in a 4% Nusieve-Seakem (3:1) blend (FMC Bioproducts) and the DNA was stained with ethidium bromide.

Table 1
Inhibitory effects of the compounds on the replication of HIV-1(HTLV-III_B) in MT-4 cells infected at a m.o.i. of 0.01[a]

Compounds	IC_{50}[b] (μM)	CC_{50}[c] (μM)
Nucleoside RT inhibitors		
Zidovudine	0.002 ± 0.001	56
Lamivudine	0.4 ± 0.2	611
Nonnucleoside RT inhibitors		
Nevirapine	0.04 ± 0.02	600
Delavirdine	0.01 ± 0.005	51
Loviride	0.009 ± 0.004	>350
Acyclic nucleoside phosphonate RT inhibitor		
Tenofovir	0.7 ± 0.3	>1740
Protease inhibitors		
Saquinavir	0.003 ± 0.0003	18
Ritonavir	0.01 ± 0.01	17
Indinavir	0.02 ± 0.005	24

[a] All data represent mean values for at least three separate experiments.

[b] A total of 50% inhibitory concentration, or concentration required to inhibit the cytopathic effect of HIV-1(HTLV-III_B) in MT-4 cells by 50%.

[c] A total of 50% cytotoxic concentration, or concentration required to cause 50% reduction of the viability of MT-4 cells.

3. Results

When tested at a m.o.i. = 0.01, all test compounds, i.e. zidovudine, lamivudine, nevirapine, delavirdine, loviride, tenofovir, ritonavir, indinavir and saquinavir, proved highly inhibitory to the replication of HIV-1(HTLV-III_B) (Table 1). Their 50% cytotoxic concentration (CC_{50}) was at least 850-fold higher than their IC_{50}. When we increased the m.o.i. from 0.01 to 1, about 10-fold higher concentrations of these compounds were required to obtain 50% inhibition of viral growth (results not shown). The IC_{90} at m.o.i. = 1 was approximately 30 times higher than the IC_{50} at m.o.i. = 0.01 for the RT inhibitors, whereas no IC_{90} at m.o.i. = 1 could be obtained at 1000 times the IC_{50} at m.o.i. = 0.01 for the protease inhibitors.

Based on these results, we conducted long-term cell culture experiments using MT-4 cells, infected with HIV-1(HTLV-III_B) at a m.o.i. = 1 in the

Table 2
Virus breakthrough upon removal of loviride

	Days post wash	Loviride used at a concentration of					
		4.5 μM ($500 \times IC_{50}$[a])		0.45 μM ($50 \times IC_{50}$)		Virus control	
		CPE[b] (%)	p24 (pg/ml)	CPE (%)	p24 (pg/ml)	CPE (%)	p24 (pg/ml)
Drug wash control (0 h[c])	1	10	4138	10	4016	10	3509
24 h[c]	1	0	65	0	109	15	3509
	2	5	729	15	3859	50	30 388
	3	25	15 682	50	33 563	100	66 678
	4	100	81 410	100	60 732	100	106 104
48 h	1	0	1	0	176	15	3509
	2	5	112	15	1568	50	30 388
	3	50	32 164	100	41 965	100	68 678
	4	100	88 250	100	94 895	100	120 104
72 h	1	0	1	5	504	15	3509
	2	0	432	50	33 806	50	30 388
	3	20	13 726	100	121 550	100	68 678
	4	100	92 374	100	137 971	100	120 104

[a] A total of 50% inhibitory concentration, or concentration required to inhibit the cytopathic effect of HIV-1(HTLV-III_B) in MT-4 cells by 50% at a m.o.i. = 0.01.
[b] Cytopathic effect, by microscopic observation.
[c] Duration of drug treatment.

presence of 50 and 500 times the IC_{50}, determined at a m.o.i. of 0.01, of the antiviral drugs. Viral growth in drug wash controls was not delayed as compared to untreated cultures (e.g. Table 2). Persistence of drug by inadequate washing could therefore be excluded. As shown in Table 2 for loviride at $500\times$ its IC_{50} (4.5 μM), treatment of HIV-infected cells for 24, 48 or 72 h was unable to prevent virus breakthrough upon removal of the compound. However, viral breakthrough was delayed for 1–2 days: the p24 level on day 1 (post wash) of the untreated virus control (i.e. 3509 pg/ml) was attained between day 2 and 3 after removal of loviride. Increasing p24 levels correlated well with microscopic observation of the cytopathic effects. A 10-fold lower concentration of loviride resulted in a more rapid viral breakthrough. The p24 level at day 2 post wash (3859 pg/ml) was similar to that in the control on day 1 post wash (3509 pg/ml).

Similar results were obtained when using nevirapine, delavirdine and lamivudine (Table 3). When we treated MT-4 cells infected at a m.o.i. = 1 with $500 \times IC_{50}$ of tenofovir for 24, 48 or 72 h, resumption of viral replication was observed with a delay of 2–3 days after removal of the compound when compared to untreated controls (Table 3).

When zidovudine and the PIs saquinavir, ritonavir and indinavir were evaluated under the same experimental conditions, none of these compounds at 500 times their IC_{50} was able to prevent or delay viral breakthrough upon removal of the compounds. When culture supernatants were checked for infectivity, we noted no significant difference in p24 to viral load ratio using different RTIs or PIs (data not shown). Viral breakthrough resulted in virus particles that all had the wild phenotype. Furthermore, HIV-1 proviral DNA was detected in HIV-infected MT-4 cells treated for 72 h at 500 times the IC_{50} of zidovudine, lamivudine, nevirapine, delavirdine, loviride and tenofovir (Fig. 1). Zidovudine, lamivudine, nevirapine, delavirdine, loviride and tenofovir were

Table 3
Time after removal of the compounds required to obtain similar p24 levels to those on day 1 of the untreated virus control

Compounds (hours)[b]	Viral breakthrough (days post wash)[a]	
	$500 \times IC_{50}$	$50 \times IC_{50}$
Nucleoside RT inhibitors		
Zidovudine		
24 h	0	0
48 h	0	0
72 h	0	0
Lamivudine		
24 h	2	1–2
48 h	1–2	0–1
72 h	1	0–1
Nonnucleoside RT inhibitors		
Nevirapine		
24 h	1–2	1–2
48 h	1–2	1
72 h	1–2	0–1
Delavirdine		
24 h	1–2	1–2
48 h	1–2	1
72 h	1–2	0–1
Loviride		
24 h	1–2	1
48 h	1–2	1
72 h	1–2	0–1
Acyclic nucleoside phosphonate RT inhibitor		
Tenofovir		
24 h	3	1
48 h	2	0–1
72 h	2	0
Protease inhibitors		
Saquinavir		
24 h	0	0
48 h	0	0
72 h	0	0
Ritonavir		
24 h	0	0
48 h	0	0
72 h	0	0
Indinavir		
24 h	0	0
48 h	0	0
72 h	0	0

[a] Similar p24 levels to those on day 1 of the untreated virus control was considered as viral breakthrough.

[b] Duration of drug treatment. Results are means for at least two separate experiments.

able to decrease the levels of proviral DNA, but, under the experimental conditions used (i.e. treatment for 24, 48 or 72 h), none of the compounds tested was able to clear the culture from HIV.

4. Discussion

None of the antivirals studied (zidovudine, lamivudine, nevirapine, delavirdine, loviride, tenofovir, ritonavir, indinavir and saquinavir) was able to prevent resumption of viral growth in MT-4 cells at a m.o.i. = 1 upon removal of the compound after a treatment period of 1, 2 or 3 days. Some RT inhibitors were able to delay viral breakthrough for several days: i.e. the acyclic nucleoside phosphonate RT inhibitor tenofovir at 350 μM was able to delay viral breakthrough for up to 3 days. The NRTI lamivudine (200 and 20 μM) and the NNRTIs nevirapine (20 and 2 μM), delavirdine (5 and 0.5 μM) and loviride (4.5 and 0.45 μM) were able to prevent viral breakthrough for maximally 2 days. However, a 72-h treatment period at these drug concentrations was not sufficient to suppress the appearance of proviral HIV-1(HTLV-III_B) DNA completely.

Balzarini et al. (1993) previously demonstrated that nevirapine and delavirdine at 100-–250-fold higher concentrations than their IC_{50} prevented viral breakthrough after 15 subcultivations (35 days). Balzarini et al. (1995) and Okamoto et al. (1996) also reported that complete clearance of HIV-1 from the virus-infected cells could be obtained by double- or triple-drug combinations of RT inhibitors in vitro. However, in all these experiments cells were infected with HIV-1(HTLV-III_B) at a m.o.i. = 0.01 and compounds were removed only after approximately 35 days. We wanted to work at a m.o.i. = 1 to obtain a synchronized infection for all the cells. That of all the compounds used tenofovir (PMPA) was the most potent compound to delay viral breakthrough might be explained by the fact that the half-life for the disappearance of PMPApp, the active form of PMPA, is 12–15 h in activated lymphocytes and 33–50 h in resting lymphocytes (Robbins et al., 1998). This long persistence of PMPApp, particularly in resting lymphocytes, is

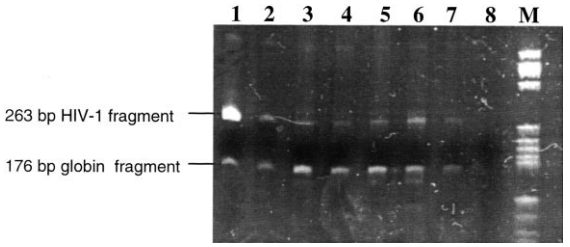

Fig. 1. Inhibitory effects of 4 μM zidovudine (2); 200 μM lamivudine (3); 20 μM nevirapine (4); 5 μM delavirdine (5); 4.5 μM loviride (6); and 350 μM tenofovir (7); on the presence of HIV-1(HTLV-III_B) proviral DNA in MT-4 cells (following a 72-h treatment period), as revealed by PCR analysis. Lanes 1 and 8, positive and negative control, respectively; M: molecular size marker.

typical for the acyclic nucleoside phosphonate analogs.

The NRTI zidovudine at 4 μM ($500 \times IC_{50}$) was not able to delay viral breakthrough upon removal of the compound. Balzarini et al. (1993) previously demonstrated that zidovudine, even when administered at a 1000-fold higher concentration than its IC_{50} failed to prevent virus breakthrough after a second subcultivation of CEM cells infected at a m.o.i. = 0.01. The PIs saquinavir (1.5 and 0.15 μM), ritonavir (5 and 0.5 μM) and indinavir (10 and 1 μM) were not at all able to delay viral breakthrough upon removal of the antiviral. These results could have been predicted from the fact that no IC_{90} at m.o.i. = 1 could be obtained at 1000 times the IC_{50} at m.o.i. = 0.01 for the PIs. We also studied the infectivity of the newly produced virus particles after treatment with PIs by calculation of the ratio of p24 to infectivity. Our data (not shown) indicated that the produced virions in the presence of PIs were as infectious as those produced in the presence of RTIs.

For those drugs that did delay virus replication, we observed a negative correlation between the delay of viral breakthrough and the duration of the treatment (Table 3). The longer the drug had been present, the more rapid virus production resumed once the antivirals were removed. This suggests that the virus replication was actually never completely inhibited.

In conclusion, the antiviral effects of the HIV-1 inhibitors (NRTIs, NNRTIs, PIs) seem to be completely reversible at the cellular level. Our findings indicate that drug holidays result in breakthrough of virus that had been suppressed for several days by adequate drug levels, and this may at least in part explain why monotherapy fails in vivo.

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