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Complete inhibition of viral breakthrough by combination of MKC-442 with AZT during a long-term culture of HIV-1-infected cells

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Abstract

We have investigated viral breakthrough during a long-term culture of HIV-1-infected cells with the non-nucleoside reverse transcriptase inhibitors (NNRTIs) 6-benzyl-1-ethoxymethyl-5-isopropyluracil (MKC-442), nevirapine and loviride (α-APA). When the compounds were examined for their inhibitory effects on HIV-1 (HE strain) replication in MT-4 cells on day 4 after virus infection, the 50% effective concentrations (EC_{50}) of MKC-442, nevirapine and loviride were 9.4, 98 and 21 nM, respectively. After a 48-day culture period, MKC-442, nevirapine and loviride completely inhibited viral breakthrough at concentrations of 1, 5 and 1 μM, respectively. These concentrations were 50–100-fold higher than their EC_{50} values. When the cells were treated with either MKC-442 (0.04 and 0.2 μM), nevirapine (0.2 and 1 μM) or loviride (0.04 and 0.2 μM) in combination with AZT (0.005 μM), only the combination of 0.2 μM MKC-442 with 0.005 μM AZT could completely inhibit the breakthrough of HIV-1 after a 68-day culture period. Polymerase chain reaction (PCR) analysis revealed that no proviral DNA was detected in the cells treated with this combination. Except for two combinations (0.04 μM MKC-442 + 0.005 μM AZT and 0.04 μM loviride + 0.005 μM AZT), all of the viruses isolated during combination treatments had various amino acid mutations in their reverse transcriptase (RT). These results indicate that the combination treatment with a relatively high dose of MKC-442 and a low dose of AZT may have potential to suppress the emergence of drug resistance during a long-term treatment in vivo and should be further pursued in HIV-1-infected patients.

Keywords: HIV-1; Non-nucleoside reverse transcriptase inhibitor (NNRTI); HEPT derivative; Drug resistance; Combination chemotherapy

1. Introduction

The first compounds shown to specifically inhibit human immunodeficiency virus type 1

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(HIV-1) were 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine (HEPT) (Baba et al., 1989; Miyasaka et al., 1989) and tetrahydroimidazo[4,5,1-*jk*][1,4]benzodiazepine-2(1*H*)-one and -thione (TIBO) derivatives (Pauwels et al., 1990). These compounds and their derivatives (Baba et al., 1991a,b) have later been classified as non-nucleoside reverse transcriptase (RT) inhibitors, together with nevirapine (Merluzzi et al., 1990) and pyridinone derivatives (Goldman et al., 1991). The non-nucleoside RT inhibitors (NNRTIs) inhibit the replication of HIV-1 at concentrations that are three to five orders of magnitude below their cytotoxic threshold. However, rapid emergence of drug-resistant mutants caused by point mutations in the RT gene is regarded as an obstacle to the clinical usefulness of the non-nucleoside RT inhibitors (for a review see De Clercq, 1994).

To circumvent this problem, combination chemotherapy of a non-nucleoside RT inhibitor with one or two of the nucleoside RT inhibitors, such as 3′-azido-3′-deoxythymidine (AZT) and 2′,3′-dideoxyinosine (ddI), has been proposed (Chow et al., 1993; Richman et al., 1994). We have recently demonstrated that the combination of the HEPT derivative 6-benzyl-1-ethoxymethyl-5-isopropyluracil (MKC-442) and either AZT, ddI or 2′,3′-dideoxycytidine (ddC) synergistically inhibits the replication of HIV-1 in vitro (Baba et al., 1994). Furthermore, the combination of MKC-442, but not of TIBO, nevirapine or pyridinone, with AZT-triphosphate (AZT-TP) also synergistically suppresses the activity of HIV-1 RT in an enzymatic assay system (Yuasa et al., 1993). These findings have prompted us to examine whether the combination of MKC-442 and AZT is effective in inhibiting the emergence of HIV-1 mutants in vitro. In this study, we have examined a long-term culture of HIV-1-infected cells in the presence of either MKC-442, nevirapine or loviride (α-APA) (Pauwels et al., 1993), with or without AZT, and found that MKC-442 prevents the breakthrough of HIV-1 most effectively when combined with a low concentration of AZT.

2. Materials and methods

2.1. Compounds

MKC-442 was prepared according to the procedure described in Tanaka et al. (1995). Nevirapine and loviride were synthesized at the Yokohama Research Center of Mitsubishi Chemical Corporation. The chemical structures of these non-nucleoside RT inhibitors are shown in Fig. 1. Purity of the compounds was checked by thin-layer chromatography on silica gel. AZT was purchased from Sigma Chemical Co. (St. Louis, MO). All compounds were dissolved in dimethyl sulfoxide (DMSO) at 50 mM (or higher) to exclude any antiviral or cytotoxic effect of DMSO and were stored at −20°C until use.

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), 100 U/ml penicillin G, and 100 μg/ml streptomycin (culture medium). Two strains of HIV-1 (HTLV-III_B and HIV-1_{HE}) were used in the experiments. HIV-1_{HE} is a clinical isolate from a Belgian AIDS patient (Pauwels et al., 1990). The virus strains were propagated in MT-4 cells. Titers of virus stocks were determined in MT-4 cells, and the virus stocks were stored at −80°C until use.

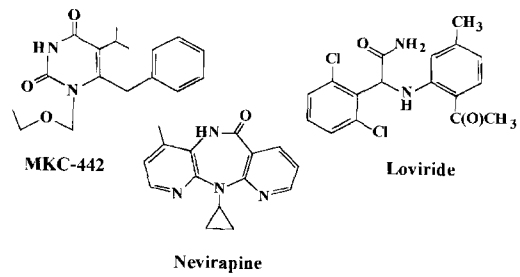

Fig. 1. Non-nucleoside RT inhibitors used in this study.

2.3. Antiviral assays

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, MT-4 cells were suspended at 1×10^5 cells/ml and infected with HIV-1 at a multiplicity of infection (m.o.i.) of 0.02. Immediately after infection, 100 μl of the cell suspension was brought into each well of a flat-bottomed microtiter tray containing various concentrations of the test compounds. After a 4-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 suspended at 1×10^5 cells/ml and infected with HIV-1_{HE} at an m.o.i. of 0.02. After a 2-h incubation for viral adsorption, the cells were washed three times with culture medium to remove unadsorbed viral particles, and 1 ml of the cell suspension was brought into each well of a 24-well tray containing various concentrations of the test compounds. After a 4-day incubation at 37°C, the cells were observed microscopically, subcultured with fresh culture medium containing appropriate concentrations of the compounds, and further incubated. At the time of subcultivation, culture supernatants were collected and examined for their p24 antigen level by using an antigen-capture ELISA kit (Cellular Products Inc., Buffalo, NY). When the cells were completely destroyed by the virus-induced CPE, a subsequent culture was not carried out. In this case, culture supernatants were collected and examined for their p24 antigen level and viral infectivity. The drug sensitivity of isolated viruses and their amino acid sequences in the RT were also determined.

2.5. Determination of nucleotide sequences of RT

Viral RNA was extracted from 200 μl of the culture supernatants using the RNAzol™ B RNA isolation reagent (Biotecx Laboratories, Inc., Houston, TX). cDNA was synthesized by adding 2.5 units of Moloney murine leukemia virus RT and 50 pmol/μl of random hexamer. The cDNA was amplified by the standard PCR method with 1 μM of the sense primer SYR1 (5′-CCAAAAGTTAAACAATGGCCATTGAC-3′) and the antisense primer SYR2 (5′-TCTGACTTGCCCAATTCAATTTCCC-3′) to obtain a 754-bp fragment involving nucleosides 2636–3389 of the HIV-1 (Ratner et al., 1985). The amplified product was isolated from 5% polyacrylamide gels, treated with T4 polymerase and T4 polynucleotide kinase, and inserted into the *Sma*I site of pUC19. More than three independent clones were isolated. The clones were analyzed for their nucleotide sequences by the PRISM™ Ready Reaction DyeDeoxy Terminator Cycle sequencing kit (Applied Biosystems, Inc., Foster City, CA) and Model 377 DNA sequencer (Applied Biosystems, Inc., Foster City, CA).

3. Results

When we evaluated MKC-442, nevirapine and loviride for their inhibitory effects on HIV-1 replication in MT-4 cells, we found that all compounds were highly potent and selective inhibitors of both the HTLV-III_B and HIV-1_{HE} strain. The EC_{50} values of MKC-442, nevirapine and loviride for these HIV-1 strains were approximately 10, 100 and 10–20 nM, respectively (Table 1). On the other hand, the 50% cytotoxic concentrations (CC_{50}) of MKC-442, nevirapine and loviride were 105, 155 and 33 μM, respectively. Thus, MKC-442 proved to be the most selective inhibitor of HIV-1 replication among the tested non-nucleoside RT inhibitors. Under the same assay conditions, the EC_{50} and CC_{50} of AZT were 3.0–3.2 nM and 4.6 μM, respectively (Table 1).

Based on the results of their anti-HIV-1 activity, we conducted the experiments on a long-term culture of HIV-1-infected MT-4 cells in the pres-

Table 1
Inhibitory effects of MKC-442, nevirapine, loviride and AZT on HIV-1 replication in MT-4 cells

Compound	Virus	EC_{50} [a] (nM)	CC_{50} [b] (μM)
MKC-442	HTLV-III_B	10 ± 2	105 ± 11
	HIV-1_{HE}	9.4 ± 2.6	
Nevirapine	HTLV-III_B	120 ± 10	155 ± 19
	HIV-1_{HE}	98 ± 3	
Loviride	HTLV-III_B	13 ± 2	33 ± 4
	HIV-1_{HE}	21 ± 9	
AZT	HTLV-III_B	3.2 ± 0.8	4.6 ± 1.2
	HIV-1_{HE}	3.0 ± 1.0	

[a] 50% Effective concentration based on the inhibition of HIV-1-induced CPE in MT-4 cells.
[b] 50% Cytotoxic concentration based on the reduction of number of viable mock-infected MT-4 cells.
All data represent means $\pm$ standard deviations for at least three separate experiments.

ence of either MKC-442, nevirapine or loviride. The concentrations used in the experiments were 0.2, 1, and 5 μM. As shown in Fig. 2, at a concentration of 0.2 μM, the p24 antigen level of the culture supernatants rapidly increased after a few passages of the infected cells (after 8–12 days) for all compounds. In contrast, the viral breakthrough was completely inhibited by the compounds at a concentration of 1 and 5 μM for MKC-442 and loviride, and 5 μM for nevirapine throughout the culture period (48 days). In these experiments, the compounds were removed from culture medium after 36 days of incubation, yet the p24 antigen level remained undetectable (< 78 pg/ml) for up to 48 days (Fig. 2). These results suggest that sufficiently high concentrations (50–100-fold higher than the EC_{50}) of non-nucleoside RT inhibitors can suppress the viral breakthrough in HIV-1-infected cells during a long-term culture period.

In the next set of experiments, we examined whether the combination of the non-nucleoside RT inhibitors (either MKC-442, nevirapine or loviride) and AZT was more effective than their single use in inhibiting the breakthrough of HIV-1. Therefore, the same and 5-fold lower concentrations of the NNRTIs (i.e. 0.2 and 0.04 μM for MKC-442 and loviride and 1 and 0.2 μM for nevirapine) were used in the experiments. The concentration of AZT was fixed at 0.005 μM, which was slightly higher than its EC_{50} value determined in the anti-HIV-1 assay (Table 1). As shown in Fig. 3, the combination of 0.2 μM MKC-442 and 0.005 μM AZT completely suppressed the viral breakthrough throughout the culture period (68 days). After 52 days of incubation, MKC-442 and AZT were removed from the culture medium, yet the p24 antigen level re-

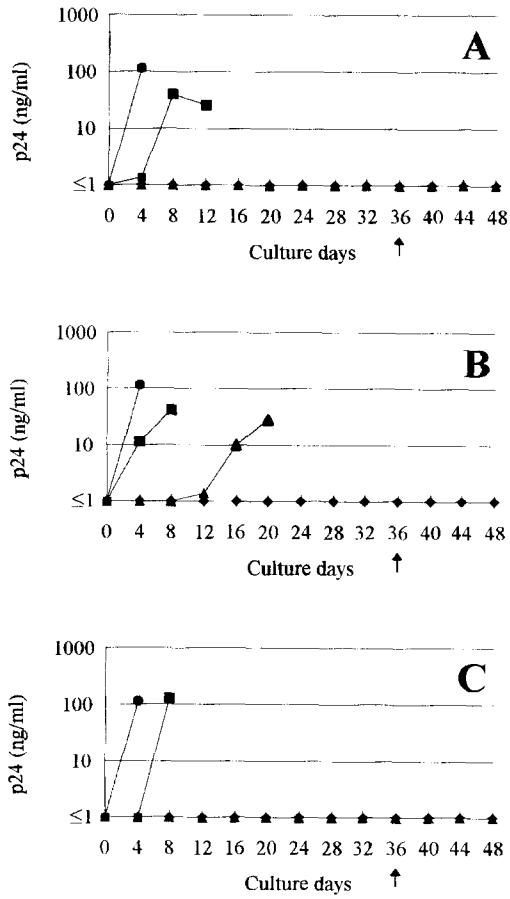

Fig. 2. Long-term culture of HIV-1-infected MT-4 cells in the presence of (A) MKC-442, (B) nevirapine and (C) loviride. The cells were infected with HIV-1_{HE} and cultured in the presence of test compounds at 0 μM (●), 0.2 μM (■), 1 μM (▲), and 5 μM (◆). Every 4 days, the cells were subcultured, and the culture supernatants were examined for their p24 antigen level. After 36 days (↑), the cells were cultured in the absence of compounds.

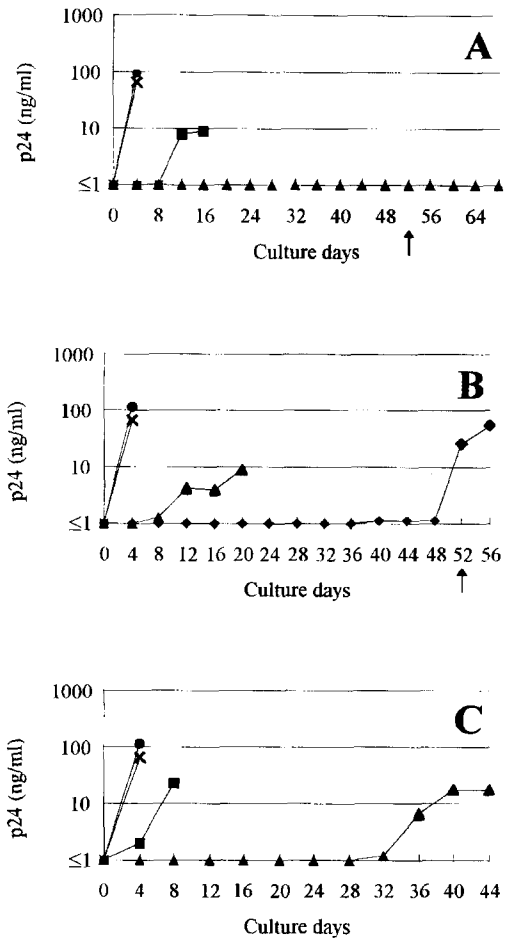

Fig. 3. Long-term culture of HIV-1-infected MT-4 cells in the presence of (A) MKC-442 + AZT, (B) nevirapine + AZT and (C) loviride + AZT. The cells were infected with HIV-1_{HE} and cultured in the presence of the test compounds at 0 μM (●), 0.04 μM (■), 0.2 μM (▲), and 1 μM (◆). The concentration of AZT alone (×) and in combination was fixed at 0.005 μM. Every 4 days, the cells were subcultured and the culture supernatants were examined for their p24 antigen level. After 52 days (↑), the cells were cultured in the absence of compounds.

mained undetectable (< 78 pg/ml) for up to 68 days. Furthermore, PCR analysis using SK-38 and SK-39 as a primer pair (Ou et al., 1988) revealed that HIV-1 proviral DNA was also undetectable in MT-4 cells after 68 days (Fig. 4). No other drug combinations tested could achieve complete inhibition of viral breakthrough (Fig. 3).

To determine whether viral breakthrough during the combination treatments could be attributed to the emergence of drug-resistant HIV-1 mutants, viral RNA was extracted from the culture supernatants and examined for its amino acid sequence in the RT. The sensitivity of breakthrough viruses to MKC-442, nevirapine, loviride and AZT was also evaluated. Except for two combinations (0.04 μM MKC-442 + 0.005 μM AZT and 0.04 μM loviride + 0.005 μM AZT), all of the viruses isolated during combination treatments had one or two amino acid mutations as compared to the wild type (Table 2). However, the only virus obtained after the treatment with 0.2 μM loviride and 0.005 μM AZT showed resistance to MKC-442 and loviride (Table 3). Other viruses did not display significant resistance to any NNRTIs examined. Furthermore, although the p24 level of culture supernatant on day 56 after the treatment with 1 μM nevirapine and 0.005 μM AZT was quite high (53 ng/ml) (Fig. 3), the infectivity of isolated virus was too low to examine its drug sensitivity (Table 3).

4. Discussion

Among the NNRTIs, MKC-442 is one of the most potent and selective inhibitors of HIV-1 replication in vitro (Baba et al., 1994). Since the NNRTIs, including MKC-442, have high specificity to HIV-1 RT, rapid emergence of drug-resistant mutants may not be avoidable during monotherapy with this class of compounds. However, recent studies have demonstrated that sufficiently high concentrations (more than 1 μg/ml) of NNRTIs are able to completely suppress the replication of HIV-1, so that drug-resistant mutants do not emerge (Balzarini et al., 1993a). In the present study, we have also shown that MKC-442 can completely inhibit the viral breakthrough during a long-term culture of HIV-1-infected MT-4 cells at a concentration of 1 μM or higher (Fig. 2A). Based on our pharmacokinetic studies of MKC-442 in animals, this concentration may be achievable in humans (data not shown).

Another important finding is that the complete suppression of viral breakthrough could be

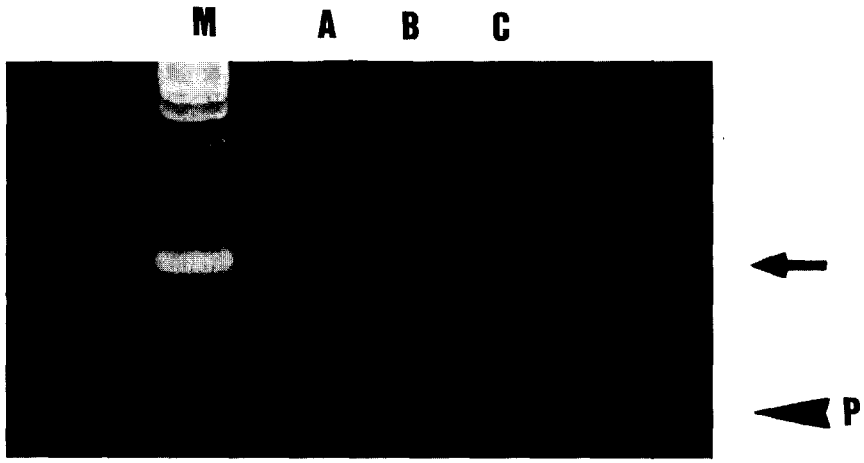

Fig. 4. Detection of HIV-1 proviral DNA by PCR. DNA was extracted from (A) mock-infected MT-4 cells, (B) HIV-1-infected MT-4 cells cultured in the absence of compounds and (C) HIV-1-infected MT-4 cells cultured in the presence of 0.2 μM MKC-442 and 0.005 μM AZT. After 52 days, the cells were cultured in the absence of compounds and DNA was extracted on day 68. DNA amplification was carried out using SK-38 and SK-39 as a primer pair, as previously described (Ou et al., 1988). P is the band of the primer pair.

achieved at a lower concentration (0.2 μM) of MKC-442 when combined with a very low concentration (0.005 μM) of AZT (Fig. 3A). In fact, AZT alone at this concentration had little, if any, influence on the p24 antigen level in culture supernatants on day 4 after virus infection (Fig. 3). Similar results were obtained in our previous study, where the combination of 0.028 μM MKC-442 and 0.008 μM AZT completely suppressed the breakthrough of HTLV-III_B strain for more than 60 days (Baba et al., 1995). In this study, however, a clinical isolate (HIV-1_{HE} strain) was used throughout the experiments, since it seemed to contain more variants than the HTLV-III_B strain. For nevirapine and loviride, the combination with AZT could significantly delay viral breakthrough as compared with their single use; nevertheless, complete suppression was not observed (Fig. 3). This difference could be explained in part by our previous observation that MKC-442, but not other tested NNRTIs in combination with AZT-TP synergistically inhibit HIV-1 RT activity in an enzymatic assay system (Yuasa et al., 1993).

Studies on HIV-1 mutants resistant to NNRTIs have proved that the $Tyr^{181} \rightarrow Cys$ mutation is associated with resistance to most of the NNRTIs, including MKC-442 (De Clercq, 1994; Balzarini et al., 1995; Seki et al., 1995). On the other hand, it has also been shown that most of the mutations other than $Tyr^{181} \rightarrow Cys$ confer resistance to only limited classes of NNRTIs (Balzarini et al., 1993b; Byrnes et al., 1993). Furthermore, the combination with nucleoside RT inhibitors, such as AZT or ddI, is known to alter the amino acid mutations responsible for resistance to NNRTIs (Chow et al., 1993; Richman et al., 1994; Baba et al., 1995). In the present study, various amino acid mutations were identified in the RT region of isolated viruses (Table 2). The only virus isolated in the presence of 0.2 μM loviride and 0.005 μM AZT, which had the $Asp^{179} \rightarrow Glu$ and $Tyr^{188} \rightarrow His$ mutations, displayed reduced sensitivity to MKC-442 as well as loviride (Table 3). The $Tyr^{188} \rightarrow His$ mutation has been reported to confer resistance to the HEPT derivatives but not to nevirapine (Balzarini et al., 1995). With respect to the viruses isolated in the presence of nevirapine and AZT, they had novel mutations ($Val^{75} \rightarrow Ile$ and $Ile^{132} \rightarrow Leu$) that have not been reported previously (Table 2); their contribution to enzyme activity and drug resistance is

Table 2
Amino acid changes in the RT of HIV-1 isolated after the drug combination treatments

Virus after treatment	Day [a]	Amino acid mutation	Resistance [b]
AZT alone [c]	4	None	(−)
MKC-442 (0.2 μM)+AZT [c]	68	No virus	
MKC-442 (0.04 μM)+AZT	16	None	(−)
Nevirapine (1 μM)+AZT	56	$Val^{75} \rightarrow Ile$, $Ile^{132} \rightarrow Leu$	N.D. [d]
Nevirapine (0.2 μM)+AZT	20	$Ile^{132} \rightarrow Leu$	(−)
Loviride (0.2 μM)+AZT	44	$Asp^{179} \rightarrow Glu$, $Tyr^{188} \rightarrow His$	(+)
Loviride (0.04 μM)+AZT	8	None	(−)

[a] On the indicated day after virus infection, viral RNA was extracted from the culture supernatant and examined for its amino acid sequence in RT.
[b] Detailed results are shown in Table 3.
[c] The concentration of AZT was fixed at 0.005 μM.
[d] EC_{50} values could not be determined because of low infectivity of the virus.

Table 3
Inhibitory effects of MKC-442, nevirapine, loviride and AZT on the replication of HIV-1 isolated after the drug combination treatments in MT-4 cells

Virus after treatment	EC_{50} [a] (nM)			
	MKC-442	Nevirapine	Loviride	AZT
Wild type	9.4 ± 2.6	98 ± 3	21 ± 9	3.0 ± 1.0
AZT alone [b]	4.2 ± 0.4	36 ± 3	11 ± 1	2.1 ± 0.3
MKC-442 (0.04 μM) + AZT [b]	13 ± 5	94 ± 36	32 ± 14	3.6 ± 0.9
Nevirapine (1 μM) + AZT	N.D. [c]	N.D.	N.D.	N.D.
Nevirapine (0.2 μM) + AZT	18 ± 1	130 ± 10	32 ± 11	3.0 ± 1.0
Loviride (0.2 μM) + AZT	450 ± 40	180 ± 100	>4,000	3.3 ± 0.5
Loviride (0.04 μM) +AZT	5.1 ± 0.7	28 ± 4	7.0 ± 0.1	1.5 ± 0.2

[a] 50% Effective concentration based on the inhibition of HIV-1-induced CPE in MT-4 cells.
[b] The concentration of AZT was fixed at 0.005 μM.
[c] EC_{50} values could not be determined because of low infectivity of the virus.
All data represent means ± standard deviations for at least three separate experiments.

unclear. Further studies, including RT assays with mutated enzymes, are needed to elucidate the biological significance of these mutations.

In conclusion, the combination treatment with a relatively high dose of MKC-442 and a low dose of AZT may have the potential to suppress the emergence of drug resistance during long-term treatment in vivo and should be further pursued in HIV-1-infected patients.

Acknowledgements

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