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Selective and Synergistic Inhibition of Human Immunodeficiency Virus Type 1 Reverse Transcriptase by a Non-nucleoside Inhibitor, MKC-442

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SUMMARY

In the search for 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine derivatives, we have found 6-benzyl-1-(ethoxymethyl)-5-isopropyluracil (MKC-442) to be a highly potent and selective inhibitor of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT). The IC_{50} value of MKC-442 for HIV-1 RT was 8 nM. MKC-442 did not inhibit HIV-1 RNase H, other RTs, or DNA polymerase α. Because its inhibitory pattern showed noncompetitive inhibition with regard to nucleotide substrates, its mode of action was considered to be allosteric inhibition. From the results of combination studies, MKC-442 was found to produce synergistic inhibition of HIV-1 RT with 3′-azido-2′,3′-dideoxythymidine (AZT) 5′-triphosphate (AZT·TP). The dose of AZT·TP required for 50% inhibition was reduced to one tenth of control in the presence of a half dose of MKC-442. Although other allosteric inhibitors (Nevirapine, L-696,229, and R82,913) had the same specificity for enzyme inhibition, they did not show synergism with AZT·TP in the combination index and synergy plot analyses. Synergistic inhibition of HIV-1 replication by MKC-442 and AZT has also been observed in HIV-1-infected MT-4 cells. These results suggest that MKC-442 is a unique inhibitor of HIV-1 RT, and combination therapy with MKC-442 and AZT could be advantageous in the treatment of acquired immune deficiency syndrome.

The transcription of viral RNA to proviral DNA by the enzyme RT is an essential step in the replication of HIV-1 (1, 2). Various compounds have been reported to be HIV-1 RT inhibitors, including nucleoside analogues and non-nucleoside analogues. Among the former compounds, AZT, DDI, and DDC have been approved for clinical use in HIV-1-infected patients (3). These agents have been shown to act on the enzyme after triphosphorylation in the infected cells (3). Non-nucleoside analogues have recently been discovered to be highly selective inhibitors of HIV-1 RT. These include HEPT (4, 5) and its derivatives (6, 7), R82,913 (8), nevirapine (9, 10), the pyridinone derivative L-696,229 (11), and bis(heteroaryl) piperazines (12). They are inactive against other retroviral RTs, including HIV-2 RT, AMV RT, and MMLV RT. Because they interact directly with HIV-1 RT, activation of the inhibitors, such as cellular phosphorylation, is not necessary for the inhibitors to display their activity (13). In addition, their mode of action has been found to be allosteric inhibition, indicating that their binding sites are different from those of nucleotide substrates. Allosteric inhibition results in a conformational change of the active site in the enzyme (14–17).

Although treatment with nucleoside analogues, such as AZT, improves the clinical symptoms and prolongs the life of patients with acquired immune deficiency syndrome (18, 19), long term administration of AZT is often limited by serious toxicities, including bone marrow suppression (20). In addition, HIV-1 with reduced AZT sensitivity has been obtained from AZT-treated patients, indicating that the emergence of drug-resistant variants will limit the efficacy of AZT (21, 22). To reduce the toxicities described above and suppress the emergence of drug-resistant virus, combination therapy is an attractive approach. In fact, combinations of various anti-HIV-1 agents have been examined for their antiviral activity (23). In this study, we have investigated the effect of MKC-442 in combination with AZT·TP on the inhibition of HIV-1 RT and have compared this effect with the effects of other non-nucleoside HIV-1 RT inhibitors.

ABBREVIATIONS: RT, reverse transcriptase; HIV-1, human immunodeficiency virus type 1; HIV-2, human immunodeficiency virus type 2; AMV, avian myeloblastosis virus; MMLV, Moloney murine leukemia virus; HEPT, 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine; MKC-442, 6-benzyl-1-(ethoxymethyl)-5-isopropyluracil; AZT, 3′-azido-2′,3′-dideoxythymidine; AZT·TP, 3′-azido-2′,3′-dideoxythymidine 5′-triphosphate; DDI, 2′,3′-dideoxyinosine; DDC, 2′,3′-dideoxycytidine; R82,913, tetrahydroimidazo[4,5,1-*jk*][1,4]benzodiazepine-2(1*H*)-thione; nevirapine, 6,11-dihydro-11-cyclopropyl-4-methyldipyrido[2,3-*b*:2′,3′-*e*][1,4]diazepine-6-one; L-696,229, 3-[2-(benzoxazol-2-yl)ethyl]-5-ethyl-6-methylpyridine-2(1*H*)-one; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; CI, combination index; BSA, bovine serum albumin; DMSO, dimethylsulfoxide.

Materials and Methods

Compounds. MKC-442 was prepared as described elsewhere.[1] AZT was purchased from Sigma Chemical Co. (St. Louis, MO). AZT·TP was synthesized from AZT by chemical phosphorylation (24). R82,913 was purchased from Pharmatech International Inc. (West Orange, NJ). Nevirapine and L-696,229 were synthesized as described elsewhere (25, 26).

Radiochemicals. [*methyl*-^{3}H]TTP (85.6 Ci/mmol), [8-^{3}H]dGTP (10.5 Ci/mmol), and [2,8,5′-^{3}H]ATP (45 Ci/mmol) were purchased from NEN/DuPont (Wilmington, DE).

Template/primers. Poly(rA)·$(dT)_{12-18}$, poly(rC)·$(dG)_{12-18}$, and activated calf thymus DNA were purchased from Pharmacia (Piscataway, NJ).

Enzymes. A recombinant HIV-1 (NL43 strain) RT consisting of equimolar amounts of two species (p64 and p51) was purchased from Eiken Kagaku (Tokyo, Japan) (27). AMV RT was purchased from Anglian Biotechnology Ltd. (Colchester, England). MMLV RT was purchased from Bethesda Research Laboratories (Gaithersburg, MD). Calf thymus DNA polymerase α was purchased from Toyobo (Tokyo, Japan).

HIV-1 RT assay. The standard assay for HIV-1 RT was performed at 37° for 30 min, in a 50 μl reaction mixture containing 50 mM Tris·HCl, pH 8.4, 0.1% Triton X-100, 10 mM $MgCl_2$, 2 mM dithiothreitol, 50 mM KCl, 0.1 mg/ml nuclease-free BSA (Worthington, Freehold, NJ), 0.5 μCi of 5 μM [*methyl*-^{3}H]TTP, 12 μg/ml poly(rA)·$(dT)_{12-18}$, 1% (v/v) test compounds dissolved in DMSO, and 0.05 unit of enzyme. In the case of dGTP incorporation, 0.5 μCi of 10 μM [8-^{3}H] dGTP and 24 μg/ml poly(rC)·$(dG)_{12-18}$ were used as substrates. The reaction was stopped by addition of 10 μl of 2 M EDTA, and 50 μl of the mixture were spotted on Whatman DE-81 filter paper circles. The circles were washed three times with 5% Na_2HPO_4, twice with H_2O, and twice with 95% ethanol before scintillation counting.

Other RT and DNA polymerase α assays. The reaction mixture for AMV RT contained 50 mM Tris·HCl, pH 8.0, 10 mM $MgCl_2$, 5 mM dithiothreitol, 30 mM KCl, 0.1 mg/ml nuclease-free BSA, 1 μCi of 10 μM [*methyl*-^{3}H]TTP, 5 μg/ml poly(rA)·$(dT)_{12-18}$, 1% (v/v) test compounds dissolved in DMSO, and 0.5 unit of enzyme. In the reaction mixture for MMLV RT, 1 mM $MnCl_2$ was used instead of $MgCl_2$. The reaction mixture for DNA polymerase α contained 50 mM Tris·HCl, pH 7.4, 6 mM $MgCl_2$, 5 mM dithiothreitol, 0.5 mg/ml nuclease-free BSA, 100 μg/ml activated calf thymus DNA, 1 μCi of 10 μM [*methyl*-^{3}H]TTP, 100 μM levels each of dGTP, dCTP, and dATP, and 0.5 unit of enzyme. The incubation conditions and the following procedures were the same as in the HIV-1 RT assay.

HIV-1 RNase H assay. Substrate for the RNase H assay was prepared by transcription of M13mp18 single-stranded DNA with *Escherichia coli* RNA polymerase, using [2,8,5′-^{3}H]ATP, following the method of Kane (28). The product, ^{3}H-labeled rA·M13 single-stranded DNA (5000 cpm), was used in a 50-μl reaction mixture containing 50 mM Tris·HCl, pH 8.0, 0.001% Triton X-100, 8 mM $MgCl_2$, 2 mM dithiothreitol, 50 mM KCl, 0.1 mg/ml nuclease-free BSA, 1% (v/v) test compound dissolved in DMSO, and 0.25 unit of HIV-1 RT. The incubation conditions and the following procedures were the same as for HIV-1 RT, but the RNase H activity was calculated as the reduction of counts from the initial input.

Antiviral assay. Anti-HIV-1 activity was assessed by the inhibition of virus-induced cytopathogenicity in MT-4 cells (29). Briefly, MT-4 cells (1×10^4 cells/well) in microtiter plates were infected with HIV-1 at a multiplicity of infection of 0.02 and were cultured in the presence of varying concentrations of the test compounds. After a 4-day incubation at 37°, the number of viable cells was measured by the MTT method (30). Cytotoxicity of the compounds was evaluated in parallel with their anti-HIV-1 activity. It was based on the viability of mock-infected MT-4 cells, as determined by the MTT method.

Synergy calculations. The multiple-drug effect was evaluated by the isobologram method, the median-effect principle, and synergy plot analysis. These analyses were carried out with a personal computer. The details of the methods have been described elsewhere (31, 32).

Results

Inhibition of enzymes. AZT·TP inhibited HIV-1 RT when TTP was used as a substrate, but it did not inhibit dGTP-dependent activity (Table 1). In contrast, MKC-442 inhibited both activities, and its IC_{50} value against dGTP-dependent activity was comparable to that of AZT·TP against TTP-dependent activity. In the comparison among non-nucleoside inhibitors, MKC-442 was shown to be the strongest inhibitor against both HIV-1 RT activities.

Although AZT·TP inhibited other retroviral RTs and calf DNA polymerase α, all non-nucleoside inhibitors tested in this study were inactive with them (Table 1). In addition to these DNA polymerases, RNase H was used to check the specificity of their inhibition. HIV-1 RT has a RNase H domain in a heterodimer subunit. We prepared a tritiated substrate to detect the activity and found that none of the RT inhibitors were active (Table 1). MKC-442 was also inactive against *E. coli* RNase H (data not shown).

[1] H. Tanaka, H. Takashima, M. Ubasawa, K. Sekiya, M. Baba, R. T. Walker, E. De Clercq, and T. Miyasaka. Synthesis and anti-HIV-1 activity of 6-benzoyl acyclouridine derivatives, as HIV-1 specific reverse transcriptase inhibitors. Manuscript in preparation.

TABLE 1
Enzyme inhibition by different compounds

Compound	IC_{50}[a]					
	HIV-1 RT		HIV-1 RNase H, M13 DNA-RNA, no template	AMV RT, TTP, poly(rA)·(dT)	MMLV RT, TTP, poly(rA)·(dT)	Calf DNA polymerase α, TTP, activated DNA
	TTP,[b] poly(rA)·(dT)[c]	dGTP, poly(rC)·(dG)				
	μM					
AZT·TP	0.007 ± 0.002	>1000	>1000	0.12 ± 0.03	0.20 ± 0.16	540 ± 390
MKC-442	0.21 ± 0.05	0.012 ± 0.002	>1000	>1000	>1000	>1000
R82,913	3.8 ± 1.1	0.015 ± 0.010	>1000	>1000	>1000	>1000
Nevirapine	2.9 ± 0.3	0.088 ± 0.032	>1000	>1000	>1000	>1000
L-696,229	0.40 ± 0.10	0.021 ± 0.015	>1000	>1000	>1000	>1000

[a] Concentration of compound required for 50% inhibition of each enzyme activity. All data represent mean values ± standard deviation for at least three separate experiments.
[b] Substrate.
[c] Template.

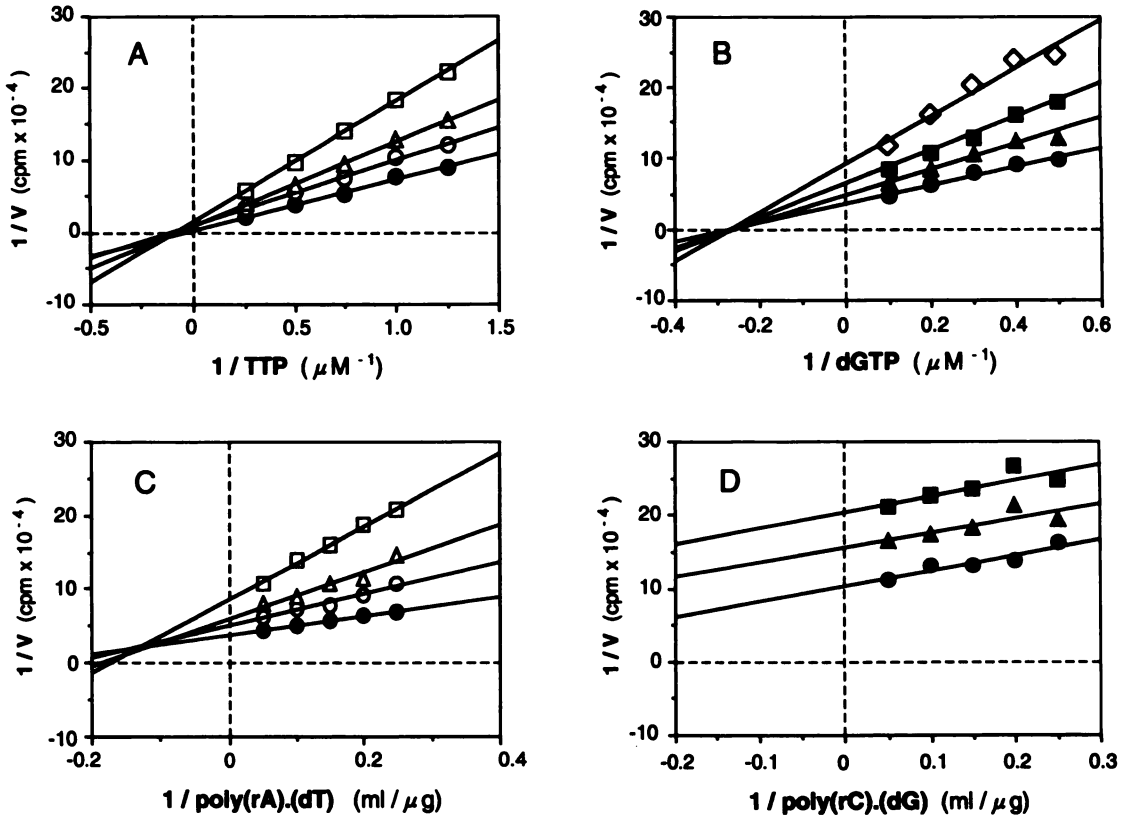

Fig. 1. Double-reciprocal plot analysis of inhibition of HIV-1 RT by MKC-442. The assay conditions were as described in Materials and Methods, except for the concentrations of substrates or template/primers. Reactions in A and B were performed with 12 μg/ml poly(rA)·(dT) (A) or 24 μg/ml poly(rC)·(dG) (B) and various concentrations of [^{3}H]TTP (A) or [^{3}H]dGTP (B). In contrast, reactions in C and D were performed with various concentrations of poly(rA)·(dT) (C) or poly(rC)·(dG) (D) and 5 μM [^{3}H]TTP (C) or 10 μM [^{3}H]dGTP (D). Concentrations of MKC-442 were 0 (●), 0.004 (▲), 0.008 (■), 0.016 (◇), 0.08 (○), 0.16 (△), and 0.32 (□) μM.

Kinetics of RT inhibition. The effect of MKC-442 on HIV-1 RT activity was examined in the presence of varying concentrations of nucleotide substrates and template/primers. As illustrated by the double-reciprocal plots in Fig. 1, A and B, MKC-442 was noncompetitive with respect to both nucleotide substrates. Under the condition of our assays, the K_m values for TTP and dGTP were 15 and 3.8 μM, respectively (Table 2). The K_i values of MKC-442 for the TTP and dGTP reactions were 0.20 and 0.010 μM, respectively. From these data, the K_i/K_m ratio for the TTP reaction was found to be 7 times higher than that for the dGTP reaction.

With regard to the template/primers, MKC-442 showed linear-mixed or uncompetitive inhibition (Fig. 1, C and D). The K_i value of MKC-442 for the poly(rC)·(dG) reaction was 0.011 μM, which was almost identical to the K_i value for the dGTP reaction (0.010 μM), in spite of the different types of inhibitory patterns. There was a similar relationship when TTP and poly(rA)·(dT) were used, that is, the K_i values were close to each other (0.2 and 0.11 μM, respectively).

Combination study for RT inhibition. Fig. 2 shows the results of combination studies with non-nucleoside inhibitors and AZT·TP in the TTP reaction. AZT·TP alone had an IC_{50} value of 7.5 nM, and the IC_{50} value of MKC-442 was 67 nM. When the combined inhibitory effects of MKC-442 and AZT·TP were analyzed by the isobologram method, a clear synergism was observed. From the curve shown in Fig. 2, when the initial dose of MKC-442 was just half of the original IC_{50} value (33.5 nM) the concentration of AZT·TP required to reach 50% inhibition was estimated as 0.75 nM, which is one tenth of the original IC_{50} value. Compared with MKC-442, the other non-nucleoside inhibitors were just additive over the wide range (30–90%) of their IC_{50} values.

TABLE 2

Inhibition constants of HIV-1 RT for MKC-442

Data represent mean values ± standard deviation for at least three separate experiments.

Fixed	Variable	K_i	K_m	K_i/K_m	Type of inhibition[a]
		μM			
Poly(rA)·(dT)	TTP	0.20 ± 0.01	14 ± 5 μM	0.014	Noncompetitive
TTP	Poly(rA)·(dT)	0.11 ± 0.01	3.4 ± 1.1 μg/ml		Linear-mixed
Poly(rC)·(dG)	dGTP	0.010 ± 0.000	5.1 ± 2.4 μM	0.002	Noncompetitive
dGTP	Poly(rC)·(dG)	0.011 ± 0.001	1.6 ± 0.5 μg/ml		Uncompetitive

[a] Type of inhibition was determined by drawing least-squares fitted lines in double-reciprocal plots (see Fig. 1).

TABLE 3

Combination study of allosteric inhibitors and AZT-TP for HIV-1 RT inhibition

Compound	AZT·TP/compound[a]	CI[b]			Synergy Plot: an analysis method % inhibition above calculated:[c] values for evaluation of synergy
		CI_{50}	CI_{70}	CI_{90}	
MKC-442	1/20	0.82 ± 0.07	0.63 ± 0.05	0.43 ± 0.10	17
R82,913	1/250	1.15 ± 0.21	1.33 ± 0.16	1.70 ± 0.07	−88
Nevirapine	1/250	1.04 ± 0.08	0.91 ± 0.12	0.69 ± 0.07	−53
L-696,229	1/40	0.97 ± 0.13	0.99 ± 0.04	1.07 ± 0.08	−119

[a] Fixed concentration ratio in mixture.

[b] CI giving 50, 70, or 90% inhibition of HIV-1 RT was calculated from four data points at the fixed concentration ratio in at least two experiments. CIs were determined under nonexclusive assumptions. A CI value of <1 would be indicative of synergy, whereas a value of 1 or >1 would be indicative of additivity or antagonism. Data represent mean ± standard deviation.

[c] The theoretical additive effect was subtracted from the observed inhibition for each combination, and the summations of 16 data points (4 × 4 concentrations) from two experiments are shown. A positive value would be indicative of synergy, whereas a value of zero or a negative value would be indicative of additivity or antagonism.

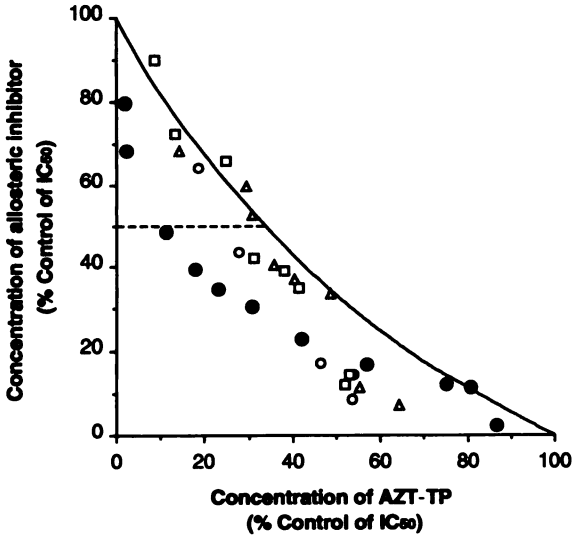

Fig. 2. Isobologram of the combined inhibitory effects of allosteric inhibitors and AZT·TP on HIV-1 RT activity. The assay conditions were as described in Materials and Methods. To summarize the results for all inhibitors, percentage values for each IC_{50} value were used on the *x*- and *y*-axes. The *curve* represents the unity line for an additive case under mutually nonexclusive assumptions. Compounds used were MKC-442 (●), R82,913 (□), nevirapine (○), and L-696,229 (△).

TABLE 4

Combination study of MKC-442 and AZT for inhibition of HIV-1 replication

AZT/MKC-442[a]	CI[b]			Synergy plot, % inhibition above calculated[c]
	CI_{50}	CI_{70}	CI_{90}	
1/8	0.60	0.40	0.21	119
2/8	0.53	0.36	0.20	80
4/8	0.60	0.39	0.21	97

[a] Fixed concentration ratio in mixture.

[b] Determined under nonexclusive assumptions, from three data points.

[c] Calculated from three data points.

Table 3 lists the CIs and the scores according to synergy plot analysis. The median-effect plots indicated that AZT·TP and MKC-442 gave parallel lines but that the line for the mixtures had a significantly greater slope than that for each agent alone (data not shown). From these data, the CIs were calculated under mutually nonexclusive assumptions (32). The synergistic effects of the combination of MKC-442 with AZT·TP were more evident when IC_{70} or IC_{90} values were used as the calculation endpoints. Furthermore, this synergism was also detected by means of the synergy plot analysis, which showed a positive value for its score. Because the CIs of the other allosteric inhibitors were very close to or more than 1.0, they were additive or antagonistic with AZT·TP. Although nevirapine had a low CI for the IC_{90}, it did not show a synergistic score in the synergy plot analysis.

Combination study for antiviral activity. A combination study was undertaken to confirm the synergistic inhibition by AZT and MKC-442 of HIV-1 replication. In this assay, AZT had an IC_{50} value of 3.2 nM and the IC_{50} value of MKC-442 was 14 nM. Table 4 reveals that both the CIs and the synergy plot scores showed very high synergism in antiviral activities. Their values were lower than those from the enzyme inhibition study (Table 3). In addition to the antiviral activity, the CIs based on the 50% cytotoxic concentrations were greater than 1.0. These results indicate that the antiviral activities of AZT and MKC-442 were enhanced in the combination study but that the cytotoxicities of both compounds were diminished.

Discussion

During the screening of HEPT derivatives as novel anti-HIV-1 agents (4, 5), MKC-442 was found to have highly potent and selective activity (33).[1] The IC_{50} values of MKC-442 and HEPT for HIV-1 in MT-4 cells were 14 nM and 7 μM, respectively. Therefore, MKC-442 was 500 times more active than HEPT. We selected this compound as one of the preclinical candidates from the results of *in vitro* efficacy as well as *in vivo* toxicity and pharmacokinetics studies.[2]

The present study indicates that MKC-442 is a highly potent and selective HIV-1 RT inhibitor and its mechanism of action is allosteric inhibition. AZT·TP is known to be a competitive inhibitor of HIV-1 RT, with respect to TTP (34). Because of their different modes of actions, we considered that the combination of AZT·TP and MKC-442 should be evaluated in an enzyme assay system. In this study, we compared synergistic effects of MKC-442 and other non-nucleoside HIV-1 RT inhibitors, such as nevirapine, L-696,229, and R82,913. In the presence of half the IC_{50} dose of MKC-442, the concentration of AZT·TP required for 50% inhibition was reduced to one tenth of its IC_{50} value. In contrast to the strong synergism of MKC-442, the other non-nucleoside inhibitors did not show obvious synergism. L-696,229 was additive and R82,913 was rather antagonistic, according to the CI. This advantage of MKC-442

[2] K. Toshida, N. Baba, M. Yamamoto, and T. Nivra, unpublished observations.

may lead to more effective combination therapy with AZT in future clinical use.

Using a virus-infected cell culture system, clear synergism of the antiviral activity of MKC-442 combined with AZT was confirmed and antagonism of their cytotoxicity was found. Almost the same results were obtained when 5-ethyl-1-ethoxymethyl-6-(phenylthio)uracil, a HEPT derivative, was used instead of MKC-442 (35). These results suggest that HEPT derivatives have the same mechanism of HIV-1 RT inhibition. It should be noted that MKC-442 and 5-ethyl-1-ethoxymethyl-6-(phenylthio)uracil, unlike AZT, have little effect on the proliferation and differentiation of mouse bone marrow cells.[3] Bone marrow suppression is the most typical and serious side effect of AZT in the treatment of patients with acquired immune deficiency syndrome (20). Combination therapy with AZT and MKC-442 would minimize these side effects.

Another advantage of combination therapy, compared with single-agent therapy, is that it may reduce the emergence of drug-resistant virus (13). To examine this possibility, long term cultivation with single or combined anti-HIV-1 agents is necessary. We are currently doing these kinds of experiments. We have recently isolated HIV-1 variants resistant to MKC-442 and have been analyzing nucleotide sequences within the RT region to detect the mutated bases. In a recent paper, it was shown that long term cultivation with AZT and DDI allowed the emergence of double drug-resistant virus after 21 days but that a triple drug combination, including AZT, DDI, and L-697,661, a close derivative of L-696,229, completely blocked viral replication after 21 days (36). Similar drug combination experiments are in progress with AZT and MKC-442. Although they are still preliminary, our recent results demonstrated that apparent suppression of the emergence of drug-resistant mutants was observed with this combination, compared with AZT alone or MKC-442 alone.[4] From our enzymological data, it could also be expected that AZT and MKC-442 would show suppression of the emergence of drug-resistant virus, even without DDI.

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[4] M. Baba, S. Yuasa, Y. Sadakata, and M. Seki, unpublished observations.

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