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Marked Inhibitory Activity of Non-nucleoside Reverse Transcriptase Inhibitors against Human Immunodeficiency Virus Type 1 When Combined with (−)2′,3′-Dideoxy-3′-thiacytidine

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SUMMARY

Human immunodeficiency virus type 1 (HIV-1)-infected CEM cells were treated (as single agents or in combination) with (minus)-2′,3′-dideoxy-3′-thiacytidine (3TC) and the following HIV-1-specific non-nucleoside reverse transcriptase (RT) inhibitors (NNRTIs): 2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-3′-spiro-5′-(4′-amino-1′,2′-oxathiole)-2′,2′-dioxide derivative of 3-methylthymidine (TSAO-m^{3}T), the thiocarboxanilides UC10 and UC42, bis(heteroaryl)piperazine (BHAP) derivative U90152, and the 1-(2-hydroxyethoxymethyl)-6-(phenylthio)thymine (HEPT) derivative 5-isopropyl-1-ethoxymethyl-6-benzyluracil (MKC-442). When used individually, the compounds led to the emergence of HIV-1 strains containing the following mutations in the RT: Glu^{138} to lysine for TSAO-m^{3}T, Met^{184} to valine for 3TC, Lys^{103} to threonine/asparagine for the thiocarboxanilides, and Tyr^{181} to cysteine for BHAP and MKC-442. When 3TC was combined with TSAO-m^{3}T, UC10, UC42, BHAP, or MKC-442, breakthrough of virus was markedly delayed or even suppressed. For these drug combinations, the concentrations of the individual drugs could be lowered by ≥25–50-fold to suppress virus breakthrough compared with the individual use of the compounds. The concomitant presence of the Lys^{138} and Ile/Val^{184} mutations was found in the RT of the mutant viruses that emerged with combination therapy of the lowest concentrations of 3TC with either the lowest concentrations of TSAO-m^{3}T or UC10 (~0.5–3-fold the EC_{50} value). These virus strains retained high sensitivity to other NNRTIs such as BHAP or HEPT. The virus mutants that arose in the presence of combinations of the lowest concentrations of 3TC with either BHAP or HEPT predominantly contained the Cys^{181} mutation in the RT. In one case, the Ile^{181} mutation was found. The latter mutations, particularly the Ile^{181} mutation, resulted in markedly decreased sensitivity to the NNRTIs but not to 3′-azido-2′,3′-dideoxythymidine or 3TC.

To be inhibitory to the RT of HIV (1–5), the ddN analogues (i.e., AZT, 2′,3′-dideoxycytidine, DDI, and 2′,3′-didehydro-2′,3′-dideoxythymidine) must be converted to their 5′-triphosphate derivatives with the use of cellular enzymes. One of the most recently reported 2′,3′-dideoxynucleoside analogues is 3TC. This heterocyclic nucleoside analogue consists of 2′-deoxycytidine, in which the 3′-carbon of the ribose ring has been replaced with a sulfur atom (6). The β-L-(minus)-isomer of 3TC has proved to be the most potent enantiomer, with low, if any, cytotoxicity (7–10). As a rule, the ddN derivatives, including 3TC, exhibit equal potency against HIV-1 and HIV-2.

In contrast, several structurally different classes of test compounds have been described as potent and selective HIV-1 inhibitors (for a review, see Ref. 11). They are targeted at a nonsubstrate binding site of the HIV-1 RT; they do not need to be metabolized to be antivirally active; and they are not inhibitory to HIV-2.

A potential drawback of the HIV-1-specific RT inhibitors

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ABBREVIATIONS: RT, reverse transcriptase; ddN, 2′,3′-dideoxynucleoside; HIV-1, human immunodeficiency virus type 1; NNRTI, non-nucleoside reverse transcriptase inhibitor; TSAO-m^{3}T, 2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-3′-spiro-5″-(4″-amino-1″,2″-oxathiole)-2″,2″-dioxide derivative of 3-methylthymidine; BHAP, bis(heteroaryl)piperazine; HEPT, 1-(2-hydroxyethoxymethyl)-6-(phenylthio)thymine; MKC-442, 5-isopropyl-1-ethoxymethyl-6-benzyluracil; AZT, 3′-azido-2′,3′-dideoxythymidine; 3TC, (−)2′,3′-dideoxy-3′-thiacytidine; DDI, 2′,3′-dideoxyinosine; $CCID_{50}$, cell culture infective dose-50.

(NNRTIs) is the rapid emergence of drug-resistant HIV-1 strains (12–15). However, the NNRTIs may maintain high activity against mutant HIV-1 strains depending on the nature of the amino acid substitutions in the RT (15, 16). A typical example is the TSAO derivatives, which predominantly select for the Glu^{138}-to-lysine mutation in the HIV-1 RT (15–18). The Lys^{138} mutant HIV-1 strains are highly resistant to TSAO derivatives but retain marked sensitivity to other NNRTIs and ddN analogues.

In contrast with other ddN derivatives and much like the NNRTIs, 3TC rapidly selects for highly resistant mutant virus strains in cell culture (19–23). 3TC predominantly selects for the Met^{184}-to-isoleucine/valine mutation in the HIV-1 RT.

In preliminary experiments, we found that NNRTIs are highly inhibitory to Met^{184}-to-isoleucine and Met^{184}-to-valine mutant virus strains and that 3TC remains highly active against mutant HIV-1 strains that contain amino acid mutations conferring resistance to the NNRTIs. These observations provided the rationale for the use of combinations of NNRTIs [i.e., TSAO, thiocarboxanilide derivatives, BHAP (U90152), MKC-442] with 3TC in attempts to improve their antiviral efficacy and prevent the emergence of drug-resistant mutant HIV-1 strains.

We found that combinations of TSAO, UC10 or UC42, and BHAP or MKC-442 with 3TC resulted in marked potentiation of the anti-HIV-1 activity and suppressed virus breakthrough in cell culture at compound concentrations that were ≥1–2 orders of magnitude lower than when the drugs were used individually.

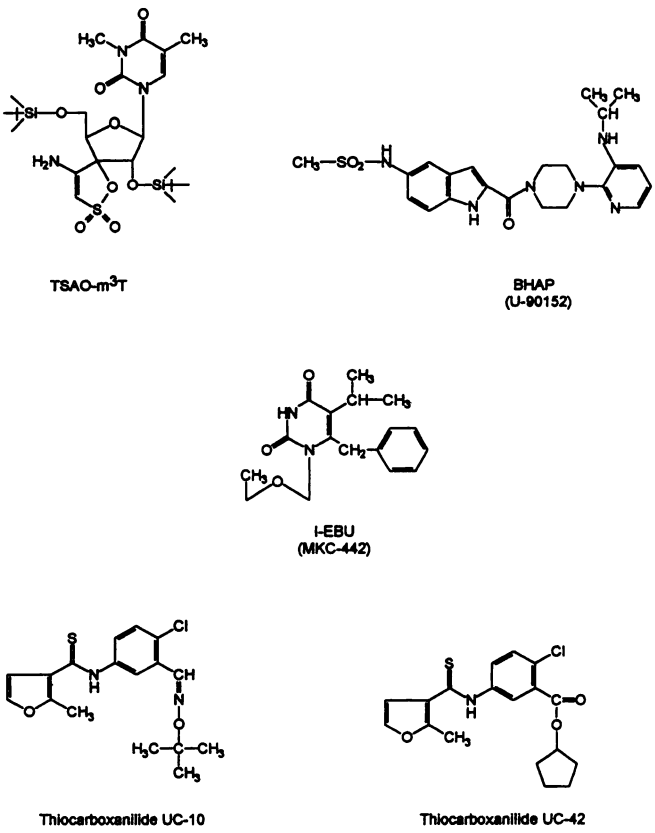

Fig. 1. Structural formulas of NNRTIs.

Materials and Methods

Test compounds. The synthesis of the TSAO derivative TSAO-m^3T has been described previously (24–26). For the origin of the thiocarboxanilide derivative UC10, see Balzarini *et al.* (27). The thiocarboxanilides UC10 and U42 were kindly provided by Uniroyal Chemical (Middlebury, CT). 3TC was kindly provided by Dr. R. Schinazi (Decatur, GA). 3TC/5′-triphosphate was a gift from Dr. J. Cameron (Glaxo-Wellcome, Wellwyne, UK). TIBO R82913 was provided by Dr. D. G. Johns and Dr. Zhang Hao (National Institutes of Health, Bethesda, MD) and obtained from Pharmatech International (West Orange, NY). Nevirapine (BI-RG-587), pyridinone (L-697,661), the BHAP derivative U90152, quinoxaline (S2720), and the HEPT derivative MKC-442 were provided by Dr. P. Ganong (Boehringer Ingelheim, Ridgefield, CT), Dr. M. Goldman (Merck, Sharp & Dohme, West Point, PA), Dr. Kirstner (Hoechst, Frankfurt, Germany), Dr. J.-P. Kleim (Hoechst), and Dr. M. Baba (Kagoshima University, Kagoshima, Japan), respectively. The structural formulas of TSAO-m^3T, the BHAP derivative U90152, MKC-442, UC42, and UC10 are given in Fig. 1. AZT, 2′,3′-dideoxycytidine, and DDI were obtained from Sigma Chemical Co. (St. Louis, MO), Dr. D. G. Johns (National Institutes of Health), and Dr. M. Hitchcock (Bristol Myers Squibb, Wallingford, CT; currently at Gilead Sciences, Foster City, CA), respectively.

Cells and viruses. CEM cells were obtained from American Type Culture Collection (Rockville, MD). HIV-1(III_B) was originally obtained from the culture supernatant of persistently HIV-1-infected H9 cells and was provided by Drs. R. C. Gallo and M. Popovic (National Institutes of Health).

Selection of HIV-1(III_B) mutant strains resistant to HIV-1-specific inhibitors. A high multiplicity of infection of HIV-1(III_B) was subjected to several passages in 5-ml CEM cell cultures (3.5×10^5 cells/ml) in the presence of fixed concentrations of 3TC (0.02, 0.05, 0.1, 0.25, 1.0, 2.5, and 10 μg/ml), TSAO-m^3T (0.02, 0.05, 0.1, 0.5, and 2.5 μg/ml), the carboxanilide derivative UC10 (0.02, 0.05, 0.1, 0.5, and 2.5 μg/ml), the BHAP derivative U90152 (0.005, 0.02, 0.04, 0.1, and 0.25 μg/ml), and the HEPT derivative MKC-442 (0.005, 0.02, 0.04, 0.1, and 0.25 μg/ml) in 25-cm^2 culture flasks (Corning, NY) to produce mutant strains that were able to grow in the presence of the individual compounds. The initial virus input into each cell culture consisted of 750 μl of supernatant that was obtained from an HIV-1-infected cell culture when the virus was most abundantly present (~4 days after infection). The culture medium consisted of RPMI-1640 containing 10% fetal bovine serum, 2 mM L-glutamine, and 0.075% $NaHCO_3$. The multiplicity of the initial infection was >500× the $CCID_{50}$. Passages were performed every 3–4 days through the addition of 0.5–1.0 ml of the infected culture supernatant to 5 ml of a suspension containing 3.5×10^5 uninfected CEM cells/ml. The supernatants of the treated HIV-1-infected cell cultures were frozen in aliquots at −70° after the syncytium formation became abundant in the cell cultures, and the virus was further passaged for at least five additional subcultivations in the presence of the test compounds.

In the drug combination experiments (3TC with either TSAO-m^3T, UC10 or UC42, and BHAP or MKC-442), the compounds were combined at the same initial concentrations as were used for single-drug treatment. Drug concentrations were not increased during subcultivations, and the treated HIV-1-infected CEM culture supernatants were frozen under similar conditions as described above. The cell cultures that did not show visible giant cell formation after 12 subcultivations were further passaged for at least an additional 10 subcultivations in the absence of the test compounds. Then, p24 determinations were performed on the culture supernatant fluids with p24 ELISA [Dupont de Nemours, Brussels (Haren), Belgium] according to the manufacturer's instructions.

Sensitivity of several HIV-1 mutant strains to various test compounds in CEM cells. CEM cells were suspended at 250,000 cells/ml of culture medium and infected with HIV-1(III_B) or the mutant HIV-1 strains at 100× the $CCID_{50}$/ml (one $CCID_{50}$ is defined

as the amount of virus that is infective to 50% of a series of identical cell cultures). Then, 100 μl of the infected cell suspensions was added to 200-μl microtiter plate wells containing 100 μl of an appropriate dilution of the test compounds. After 4 days' incubation at 37°, the cell cultures were examined for syncytium formation. Syncytium formation in the treated HIV-1-infected and untreated HIV-1-infected cell cultures was assessed through microscopic examination. Quantification of giant cell formation was made by assigning an individual score to each culture on a scale with at least eight gradations. Control HIV-1-infected cell cultures contained ~200 giant cells per microscopic view. The EC_{50} was determined as the compound concentration required to inhibit syncytium formation by 50%.

Preparation of mutant HIV-1-infected CEM cell cultures for polymerase chain reaction analysis and sequencing of the *pol* gene of the mutant HIV-1 strains. CEM cells infected with the HIV-1 mutant strains were incubated for 3 days, centrifuged, washed twice with phosphate-buffered saline, and suspended in 10 μl polymerase chain reaction buffer [10× concentration: 100 mM Tris·HCl, pH 8.3, 800 mM KCl, 15 mM $MgCl_2$, and 0.01% (w/v) gelatin (Cetus-Vanderheyden, Brussels, Belgium), 8 μl $MgCl_2$ (25 mM), 72 μl Milto-Q water, 10 μl proteinase K (10 μg) (Calbiochem) in 0.5% Tween-20, and 0.5% NP-40 in H_2O]. The cell suspension was then incubated at 56° for 1 hr and subsequently heated at 95° for 10 min. Amplification of proviral DNA (35 cycles) was performed in 10 mM Tris·HCl, pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 2.5 units of thermostable DNA polymerase (Dyna Zyme, Finnzymes), and 15 μM concentrations of all primers in a final volume of 100 μl. The primer (5′-GTAGAATTCTGTTGACTCAGATTGG and 5′-TTCTGCCAGTTCTAGCTCTGCTTCT) gave a 900-base pair product of the proviral RT gene. When no proviral DNA could be detected, a new 35-cycle polymerase chain reaction with a second set of primers (5′-CCTGAAAATCCATACAATACTCCAGTATTTG and 5′-AGTGCTTTGGTTCCTCTAAGGAGTTTAC) was used, giving a 727-base pair RT fragment covering the amino acid residues 50–270. The polymerase chain reaction products were purified from a 1% low-melting agarose gel with the use of Magic PCR Preps (Promega), directly sequenced with a Taq Dye Deoxy Terminator sequencing kit (Applied Biosystems), and analyzed with a model 373A DNA sequencer (Applied Biosystems).

Results

Drug sensitivity of HIV-1 mutant strains selected for resistance to 3TC and different HIV-1-specific RT inhibitors. Marked differences were observed in the sensitivity of various HIV-1 mutant strains to 3TC, TSAO-m^3T, and the thiocarboxanilides UC10 and UC42 (Table 1). A 3TC-resistant HIV-1 strain containing the Met^{184}-to-valine mutation in its RT proved to be highly resistant to 3TC (50% effective concentration: >50 μg/ml) but remained fully sensitive to the inhibitory effect of the NNRTIs TSAO-m^3T, UC10 and UC42, the BHAP derivative U90152, and the HEPT derivative MKC-442. In contrast, the HIV-1 mutant strains selected for resistance to TIBO R82150 (HIV-1/Leu^{100} to isoleucine), TIBO R82913 (HIV-1/Lys^{103} to asparagine), nevirapine (HIV-1/Val^{106} to alanine), TSAO-m^3T (HIV-1/Glu^{138} to lysine), pyridinone (HIV-1/Tyr^{181} to cysteine), and HEPT (HIV-1/Tyr^{188} to leucine) retained marked sensitivity to 3TC but showed a varying sensitivity/resistance pattern to the NNRTIs depending on the nature of the amino acid substitution in their RT. For example, the Leu^{100}-to-isoleucine HIV-1 mutant strain proved highly sensitive to TSAO-m^3T and UC10 (EC_{50}, 0.04–0.08 μg/ml) but had a 35-fold decreased sensitivity to UC42 (EC_{50}, 0.17 μg/ml) and substantially lost sensitivity to BHAP (EC_{50}, 0.46 μg/ml) (Table 1). The HIV-1 RT Glu^{138}-to-lysine, Tyr^{181}-to-cysteine, and Val^{106}-to-alanine mutant strains lost most of their sensitivity to TSAO-m^3T (EC_{50}, 2.0–>50 μg/ml) but retained marked sensitivity to the inhibitory effects of UC10 and UC42 (EC_{50}, 0.03–0.13 μg/ml). The Tyr^{188}-to-leucine HIV-1 mutant showed little or no sensitivity to TSAO-m^3T (EC_{50}, 50 μg/ml), UC42 (EC_{50}, 2.5 μg/ml), and MKC-442 (EC_{50}, 5.0 μg/ml) and reduced sensitivity to UC10 (EC_{50}, 0.65 μg/ml).

Thus, the NNRTI derivatives TSAO, UC10, UC42, BHAP, and MKC-442 remained fully inhibitory to the Met^{184}-to-valine mutant virus (which was resistant to 3TC), whereas 3TC retained full activity against all mutant virus strains that were selected for resistance to the NNRTIs.

As a rule, the NNRTIs were poorly cytostatic against CEM cell proliferation. Their 50% cytostatic concentrations ranged from ~5 μg/ml (for UC10 and UC42) to 100 μg/ml for TSAO-m^3T (Table 1). Thus, the selectivity indexes (ratio of 50% cytostatic concentration to EC_{50}) of the test compounds ranged from 100 to >20,000.

Selection of mutant HIV-1(III_B) strains resistant to 3TC and the NNRTIs TSAO-m^3T, UC10, UC42, the BHAP derivative U90152, and MKC-442. HIV-1(III_B)-infected CEM cell cultures were treated with 3TC, TSAO-m^3T, UC10, UC42, BHAP, and MKC-442 at fixed concentrations. At the two lowest concentrations used for 3TC, TSAO-m^3T, UC10, BHAP, and MKC-442 (i.e., at compound concentrations approximating the EC_{50} value for virus-induced cytopathicity in CEM cell cultures), virus breakthrough was evident at 4–6 days after infection and rapidly resulted in full

TABLE 1

Inhibitory effects of 3TC, TSAO, the thiocarboxanilides UC10 and UC42, the BHAP derivative U90152, and the HEPT derivative MKC-442 on mutant HIV-1 replication in CEM cells

Compound	EC_{50}[a] Mutant HIV-1(III_B) strains containing in their RT these amino acid changes								CC_{50}[b]
	III_B	100 Leu → Ile	103 Lys → Asn	106 Val → Ala	138 Glu → Lys	181 Tyr → Leu	188 Tyr → Leu	184 Met → Val	
					μg/ml				
3TC	0.04	0.05	0.03	0.01	0.018	0.01	0.025	>50	>100
TSAO-m^3T	0.024	0.042	0.138	2.2	>50	2.0	≥50	0.019	≥100
UC10	0.05	0.085	≥1	0.130	0.075	0.075	0.65	0.007	5.8
UC42	0.005	0.170	0.70	0.040	0.035	0.045	2.5	0.003	4.3
BHAP	0.006	0.46	0.27	0.16	0.016	0.25		0.003	≥20
MKC-442	0.001	0.007	0.38	0.068	0.018	0.067	5.0	0.002	≥20

[a] 50% Effective concentration, or compound concentration required to inhibit HIV-1-induced giant cell formation in CEM cell cultures by 50%.
[b] 50% Cytostatic concentration, or compound concentration required to inhibit CEM cell proliferation by 50%.

cytopathicity in the drug-treated HIV-1-infected cell cultures. At a 4–10-fold higher concentration than their EC_{50}, the drugs delayed virus appearance by one to four subcultivations. When the initial 3TC, TSAO-m^3T, BHAP, and MKC-442 drug concentrations were increased (i.e., 25–125-fold the EC_{50} for 3TC, 30–300-fold the EC_{50} for TSAO-m^3T, and 25–50-fold the EC_{50} for BHAP and MKC-442), virus breakthrough could be further delayed, although not completely suppressed. In contrast, at 20-fold the EC_{50} for UC10 and 100-fold the EC_{50} for UC42, virus breakthrough could be prevented for 14 subcultivations in the presence of the test compounds. On further subcultivations in the absence of UC10 and UC42, virus-specific cytopathicity no longer occurred. The cultures were negative for p24 and proviral DNA. Thus, at a concentration of 0.5–1.0 μg/ml, UC10 and UC42 succeeded in knocking-out the virus and clearing the HIV-1-infected CEM cell cultures from HIV-1 (Table 2).

Effects of combinations of 3TC with TSAO-m^3T, UC10 or UC42, the BHAP derivative U90152, and MKC-442 on the emergence of drug-resistant virus strains. In a preliminary experiment at a concentration of 0.1 or 0.5 μg/ml, 3TC was combined with UC42 (0.04 or 0.1 μg/ml) or TSAO-m^3T (0.4 μg/ml). Under these experimental conditions, the appearance of HIV-1-induced cytopathicity could be completely prevented. Moreover, when the HIV-1-infected CEM cell cultures that had been treated with these drug combinations at the indicated concentrations were further subcultivated in the absence of the compounds for ≥10 additional passages, no signs of virus-induced cytopathicity became evident. The CEM cell cultures not only failed to eventually develop syncytia but also failed to produce any detectable HIV-1 p24 antigen and did not reveal any detectable proviral DNA in their genome (data not shown). Thus, when the compounds were combined, their concentrations could be lowered by >25-fold (3TC and TSAO-m^3T) or >6.2-fold (UC42) to efficiently suppress virus breakthrough.

In further experiments, the combinations of 3TC with TSAO-m^3T and of 3TC with the thiocarboxanilide derivative UC10 but also of 3TC with BHAP and of 3TC with MKC-442 were investigated in greater detail. Three different 3TC concentrations (i.e., 0.02, 0.05, and 0.1 μg/ml) were combined with three different TSAO (0.05, 0.1, and 0.4 μg/ml) or UC10 (0.02, 0.05, and 0.2 μg/ml) concentrations or five different BHAP or MKC-442 (0.005, 0.02, 0.04, 0.1, and 0.25 μg/ml) concentrations. When 3TC at 0.02 μg/ml was combined with TSAO-m^3T at 0.1 μg/ml, virus breakthrough could be delayed until day 19 after infection (Table 3). At these concentrations, the individual compounds could not prevent virus breakthrough by >6 days. The combination of 3TC at 0.05 or 0.1 μg/ml (i.e., 25–50-fold lower than the 3TC concentration required to delay HIV-1 breakthrough for 16 days) with TSAO-m^3T at 0.1 or 0.4 μg/ml (i.e., 25–100-fold lower than the TSAO-m^3T concentration required to delay HIV-1 breakthrough for 17 days) led to complete suppression of virus-induced cytopathicity for ≥52 days (Table 3). Thus, combinations of 3TC with TSAO-m^3T proved to be strongly synergistic in preventing virus breakthrough.

The cell cultures that had been treated with TSAO-m^3T (0.4 μg/ml) combined with 3TC (0.05 μg/ml) or with TSAO-m^3T (0.1 μg/ml) combined with 3TC (0.1 μg/ml) showed breakthrough of virus-induced cytopathicity soon after the removal of the drugs. However, the cell cultures treated with 3TC at 0.1 μg/ml combined with TSAO-m^3T at 0.4 μg/ml were found to be negative for p24, and no viral (gp120) antigen expression was observed (even after continued subcultivation of the cells in the absence of the test compounds for an additional 12 passages).

The virus strains that emerged in the presence of the test compounds were analyzed for RT amino acid substitutions (Table 4). The TSAO-m^3T-resistant virus strain, which emerged in the presence of 0.4 μg/ml TSAO-m^3T, showed the Glu^{138}-to-lysine mutation in its RT. Lower TSAO-m^3T concentrations did not give rise to mutated virus. The virus that emerged in the presence of 3TC at 2.5 μg/ml had the Met^{184}-to-valine mutation. The lower 3TC concentrations (0.02 and 0.05 μg/ml) did not select for mutated virus. However, when TSAO-m^3T at 0.05 μg/ml was combined with 3TC at 0.05 or 0.1 μg/ml, the Ile^{184} or Val^{184} mutation appeared in the RT of the emerging virus strains. At higher TSAO-m^3T concentrations (0.1 μg/ml) in the presence of 3TC at 0.05 μg/ml, a double-mutated virus strain containing Lys^{138} and Ile^{184} was selected (Table 4).

When 3TC was combined with the thiocarboxanilide derivative UC10, both at a concentration of 0.02 μg/ml, breakthrough of virus-induced cytopathicity could be markedly delayed for 14 days compared with 4 days when the drugs were used alone (Table 3). At the relatively low concentration of 0.05 μg/ml, at which the compounds singly could not prevent virus breakthrough for >6 days, the compounds UC10 and 3TC combined prevented virus breakthrough for ≥52 days. On removal of the drugs, virus breakthrough (50% cytopathicity) occurred 14 days later (Table 3). More strikingly, when UC10 at 0.05 or 0.2 μg/ml was combined with 3TC at 0.1 μg/ml, the cells were apparently cleared of virus because no cytopathicity and no p24 production could be detected even after the drugs had been removed (at day 52 of

TABLE 2
Suppression of HIV-1-induced cytopathicity in CEM cell cultures

Compound	Concentration	Mean day of HIV-1 (50% cytopathicity) breakthrough
	μg/ml	*day*
3TC	0.02	4
	0.05	5
	0.1	13
	0.5	16
	2.5	17
TSAO-m^3T	0.05	4
	0.1	5
	0.4	13
	1.0	13
	2.5	16
	10	17
UC10	0.02	5
	0.05	6
	0.2	21
	1.0	>>50
UC42	0.04	16
	0.10	16
	0.25	50
	0.5	>>50
BHAP derivative U90152	0.005	4
	0.02	5
	0.04	7
MKC-442	0.005	5
	0.02	16
	0.04	17

TABLE 3
Effect of 3TC and NNRTI combinations on HIV-1 breakthrough in CEM cell cultures

NNRTI (μg/ml)	3TC [mean day of HIV-1 (50% cytopathicity) breakthrough]					
	0	0.02	0.05	0.1	0.5	2.5
			μg/ml (day)			
0	3 (<3)[a]	4 (<4)	6 (<4)	13 (4)	16 (12)	16 (13)
TSAO-m^{3}T						
0.05	4 (2)	6 (<4)	26 (4)	30 (22)	ND	ND
0.1	6 (<4)	19 (4)	34 (25–28)	>52[b]	ND	ND
0.4	13 (7)	23 (18)	>52[b]	>52[c]	ND	ND
1.0	14 (10)	ND	ND	ND	ND	ND
2.5	16 (12)	ND	ND	ND	ND	ND
10	17 (13)	ND	ND	ND	ND	ND
UC10						
0.02	4 (<4)	14 (4)	26 (4–21)	30 (25)	ND	ND
0.05	6 (<4)	33 (7–25)	>52[d] (63)	>52[c]	ND	ND
0.2	21 (14)	42 (32)	>52[c]	>52[c]	ND	ND
BHAP						
0.005	4 (<4)	6 (<4)	11 (<4)	20 (7–14)	ND	ND
0.02	6 (<4)	27 (4)	34 (28–32)	47 (39)	ND	ND
0.04	8 (<4)	29 (21–25)	64 (62)	>52[c]	ND	ND
0.1	16 (12)	ND	ND	ND	ND	ND
0.25	19 (13)	ND	ND	ND	ND	ND
MKC-442						
0.005	5 (<4)	7–18 (<4)	35–42 (25–32)	46 (39)	ND	ND
0.02	15 (4)	33 (28)	>52[c]	>52[c]	ND	ND
0.04	15 (11)	50 (28–32)	>52[c]	>52[c]	ND	ND
0.1	16 (13)	ND	ND	ND	ND	ND
0.25	28 (24)	ND	ND	ND	ND	ND

[a] Values in parentheses represent the day of cultivation at which virus breakthrough became evident by the appearance of a few syncytia in the cultures. These early signs of initial virus breakthrough correspond with an increase of p24 levels that proved to be at least 5-fold higher than background (~1 pg p24/ml).
[b] When the drugs were washed out at day 52 after infection and the cell cultures were further passaged in the absence of the test compounds, virus breakthrough (50% cytopathicity) occurred on day 57.
[c] Cell culture remained p24 negative, even after removal of the drugs and continued cultivation of the cells in the absence of the test compounds for an additional 12 subcultivations.
[d] When the drugs were washed out at day 52 after infection and the cell cultures were further passaged in the absence of the test compounds, virus breakthrough (50% cytopathicity) occurred at day 66.
ND, not determined.

the experiment) and cultivation was continued for ≥12 additional passages (Table 3). Thus, like the combination of 3TC with TSAO-m^{3}T, the combination of 3TC with UC10 proved to be highly synergistic in suppressing HIV replication in CEM cell cultures.

The amino acid substitutions in the RT of the virus strains that emerged in the presence of combinations of 3TC with UC10 were also determined. Although the Lys^{138} or Thr^{103} mutation appeared in the presence of the combination of 3TC at a concentration of 0.02 μg/ml with UC10 at 0.02–0.2 μg/ml, combinations of higher 3TC concentrations (i.e., 0.05 or 0.1 μg/ml) with UC10 at 0.02 or 0.05 μg/ml resulted in double-mutated virus (i.e., Lys^{138} plus Ile^{184} mutations) in the presence of 3TC at 0.05 μg/ml and of UC10 at 0.02 or 0.05 μg/ml and in the Val^{184} mutation in the presence of 3TC at 0.1 μg/ml and of UC10 at 0.02 μg/ml (Table 4). Treatment of the HIV-1-infected cells with 3TC as a single drug at 0.1, 0.5, and 2.5 μg/ml resulted in the Val^{184} mutation (Table 4).

As observed for TSAO and UC10, combination of 3TC with BHAP or MKC-442 also resulted in a marked delay of virus breakthrough at relatively low concentrations of the individual test compounds (i.e., 0.02 μg/ml 3TC combined with 0.02 μg/ml MKC-442 or BHAP, or 0.05 μg/ml 3TC combined with 0.005 μg/ml MKC-442 or 0.02 μg/ml BHAP) (Table 3). Again, complete suppression of virus breakthrough could be obtained in the combination experiments at compound concentrations that were ≥10–50-fold lower than that observed when the drugs were used as single agents. In contrast with the combinations of the lower concentrations of 3TC with the lower concentrations of TSAO-m^{3}T or UC10, combinations of 3TC with BHAP and MKC-442 did not result in the emergence of double-mutant virus strains. Either Val^{184}, Ile^{184}, or Cys^{181} RT-mutated virus strains predominantly emerged with combination therapy or at the highest BHAP or MKC-442 concentrations with single-drug therapy. Interestingly, in one case, Ile^{181} RT-mutated virus occurred in the presence of 0.04 μg/ml MKC-442 when combined with 0.02 μg/ml 3TC (Tables 3 and 4).

Triple-drug combinations in HIV-1-infected CEM cell cultures. Triple-drug combinations were used in a separate experiment contrasting the use of 3TC plus TSAO-m^{3}T or 3TC plus UC42 with the use of BHAP and MKC-442 (Table 5). Under conditions where the single drugs were unable to prevent virus breakthrough within 4–13 days after initiation of the experiment, triple-drug combination of the drugs listed in Table 5 fully prevented any virus breakthrough within 10 subcultivations (day 35). After the 10th subcultivation, the cell cultures were further incubated in the absence of the test compounds for an additional four subcultivations. Under these experimental conditions, the cell cultures that had been subjected to triple-drug combination treatment were p24 negative, and proviral DNA could not be detected in the cell extracts.

Inhibitory effects of 3TC and NNRTIs on mutant HIV-1(III$_B$)-induced syncytium formation in CEM cells. Several HIV-1-specific RT inhibitors were evaluated

TABLE 4

Amino acid mutations in HIV-1 strains emerging under combination therapy of 3TC with NNRTIs

NNRTI (μg/ml)	3TC					
	0	0.02	0.05	0.1	0.5	2.5
			μg/ml			
0	Wild-type	Wild-type	Wild-type	Val^{184}	Val^{184}	Val^{184}
TSAO-m^3T						
0.05	Wild-type	Wild-type	Ile^{184}	Ile^{184}/Val	ND	ND
0.1	Wild-type	Lys^{138}	Lys^{138} + Ile^{184}	Ile^{184}	ND	ND
0.4	Lys^{138}	Lys^{138}	Lys^{138}[a]	No virus[b]	ND	ND
UC10						
0.02	Wild-type	Lys^{138}	Lys^{138} + Ile^{184}	Val^{184}	ND	ND
0.05	Wild-type	Lys^{138}	Lys^{138} + Ile^{184}[c]	No virus	ND	ND
0.2	Wild-type	Thr^{103}	No virus	No virus	ND	ND
BHAP						
0.005	ND	Wild-type	Val^{184}	Val^{184}	ND	ND
0.02	ND	Wild-type	Ile^{184}	Ile^{184}	ND	ND
0.04	Tyr^{181}/Cys	Cys^{181}	ND	No virus	ND	ND
MKC-442						
0.005	Wild-type	Lys^{138}	Val^{184}	Val^{184}	ND	ND
0.02	ND	Cys^{181}	No virus	No virus	ND	ND
0.04	Cys^{181}	Ile^{181}	No virus	No virus	ND	ND

[a] When the drugs were washed out at day 52 after infection and the cell cultures were further passaged in the absence of the test compounds, virus breakthrough (50% cytopathicity) occurred on day 57.

[b] Complete suppression of virus breakthrough. Cell culture remained p24 negative, even after removal of the drugs and continued cultivation of the cells in the absence of the test compounds for an additional 12 subcultivations.

[c] When drugs were washed out at day 52 after infection and the cell cultures were further passaged in the absence of the test compounds, virus breakthrough (50% cytopathicity) occurred on day 66.

ND, not determined.

TABLE 5

Inhibitory effect of triple combinations of 3TC with NNRTIs on virus breakthrough in CEM cell cultures

Compound	Mean day of HIV-1 (50% cytopathicity) breakthrough	Initial day of appearance of the first syncytia in the cell cultures (p24 culture fluid levels ≥5-fold background) (1 pg p24/ml)
	day	
3TC (0.1 μg/ml)	11	<4
TSAO-m^3T (0.4 μg/ml)	13	10
UC42 (0.04 μg/ml)	15	9
BHAP (0.04 μg/ml)	9	4
MKC-442 (0.04 μg/ml)	16	13
3TC + TSAO-m^3T + BHAP	>45	>45
3TC + TSAO-m^3T + MKC-442	>45	>45
3TC + UC42 + BHAP	>45	>45
3TC + UC42 + MKC-442	>45	>45

for their inhibitory effect on wild-type HIV-1(III_B) and mutant HIV-1(III_B) strains that had been selected in HIV-1(III_B)-infected CEM cell cultures in the presence of several combinations of 3TC with TSAO-m^3T or UC10 (Table 6). The Lys^{138} RT-mutated virus strains that had emerged in the presence of TSAO-m^3T at 0.4 μg/ml (with or without 3TC at 0.05 μg/ml) showed comparable EC_{50} values for all test compounds. They retained marked sensitivity to AZT, 3TC, and all NNRTIs, except for TSAO-m^3T. Also, the Ile^{184}, Val^{184}, and Ile/Val^{184} virus mutant strains remained highly sensitive to all NNRTIs (including TSAO-m^3T) but showed a markedly reduced sensitivity for 3TC. Interestingly, the Ile^{184} virus mutant still retained some sensitivity to 3TC, whereas the Val^{184} mutant had virtually lost all sensitivity. The EC_{50} value of 3TC for the mixed Ile/Val^{184} RT HIV-1 mutant was between the EC_{50} values of 3TC for the Ile^{184} RT and those for Val^{184} RT HIV-1 strains. The mutant virus strain that contained both the Lys^{138} and Val^{184} mutations in its RT remained markedly sensitive to all NNRTIs tested, except for TSAO-m^3T. It also showed resistance to 3TC. The Thr^{103} virus mutant showed a 2–20-fold reduced sensitivity to the compounds tested (Table 6). The Cys^{181} virus mutant generally showed a reduced sensitivity to most, if not all, NNRTIs but not to AZT and 3TC. The Ile^{181} virus mutant showed markedly reduced sensitivity against all NNRTIs (usually 200-1000-fold). Again, AZT and 3TC maintained a pronounced inhibitory effect against this virus strain.

Discussion

Among the different classes of HIV RT inhibitors, the TSAO derivatives (i.e., TSAO-m^3T) and 3TC consistently selected for identical mutant HIV-1 strains that contained either the Lys^{138} (for TSAO) or the Ile/Val^{184} (for 3TC) amino acid substitution in the RT. These amino acid substitutions are located in two functionally different regions of the RT. Moreover, the Lys^{138} mutant, although resistant to TSAO-m^3T, is sensitive to 3TC, and the Ile/Val^{184} mutant, although resistant to 3TC, is sensitive to TSAO-m^3T. Also, the 3TC- and TSAO-m^3T-resistant virus mutants are still sensitive to other 2′,3′-dideoxynucleoside inhibitors, such as AZT, and to other NNRTIs, such as BHAP, nevirapine, TIBO, pyridinone, HEPT, and quinoxaline.

As demonstrated by the current findings, paired combinations of 3TC with an NNRTI (e.g., TSAO-m^3T, thiocarboxanilides, BHAP, or MKC-442) effected a marked suppression of HIV replication in CEM cell cultures. Moreover, the virus mutant strains that eventually emerged, if the drug concentrations in the mixtures were apparently too low to keep all virus suppressed, contained either no mutations in their RT (wild-type) or the predictable amino acid changes in their RT

TABLE 6
Sensitivity of mutant HIV-1 strains to test compounds

Compound	EC_{50}								
	Wild-type[a]	Lys^{138}[b]	Ile^{184}[c]	Val^{184}[d]	Ile^{184}/Val[e]	Thr^{103}[f]	Lys^{138}[g] + Ile^{184}	Cys^{181}[h]	Ile^{181}[i]
					μg/ml				
MKC-442	0.002	0.010	0.002	0.001	0.0035	0.043	0.008	0.13	2.0
TIBO R82913	0.021	0.117	0.020	0.011	0.020	0.35	0.045	0.20	4.0
Nevirapine	0.027	0.057	0.021	0.070	0.033	0.60	0.023	2.9	>5
Pyridinone	0.017	0.048	0.013	0.007	0.013	0.033	0.060	1.5	≥5
BHAP U90152	0.006	0.015	0.006	0.003	0.006	0.038	0.012	0.14	0.15
TSAO-m^3T	0.021	≥5	0.025	0.012	0.028	0.40	≥5	0.25	>5
UC10	0.009	0.065	0.009	0.008	0.013	0.30	0.042	0.042	2.0
Quinoxaline	0.001	0.002	0.001	0.003	0.001	0.012	0.003	0.013	0.26
3TC	0.008	0.026	0.70	≥5	2.7	0.013	0.45	0.01	0.043
AZT	0.006	0.005	0.007	0.004	0.004	0.010	0.005	0.004	0.011

[a] Wild-type virus isolated from the HIV-1-infected cell cultures treated with the combination of UC10 (0.05 μg/ml) and 3TC (0.05 μg/ml) and found to contain no mutations in the 50–270-amino acid domain of RT.
[b] Mutant virus strains that emerged under therapy with the combination of TSAO-m^3T (0.4 μg/ml) + 3TC (0.05 μg/ml) or with TSAO-m^3T (0.4 μg/ml) as a single drug.
[c] Mutant virus strain that emerged under therapy with the combination of TSAO-m^3T (0.1 μg/ml) + 3TC (0.1 μg/ml).
[d] Mutant virus strains that emerged under therapy with the combinations of UC10 (0.02 μg/ml) + 3TC (0.1 μg/ml) or with 3TC (2.5 μg/ml) as a single drug.
[e] Mutant virus strain that emerged under therapy with the combination of TSAO-m^3T (0.05 μg/ml) + 3TC (0.1 μg/ml).
[f] Mutant virus strain that emerged under therapy with the combination of UC10 (0.2 μg/ml) + 3TC (0.02 μg/ml).
[g] Mutant virus strains that emerged under therapy with the combination of UC10 (0.02 μg/ml) + 3TC (0.05 μg/ml) or with TSAO-m^3T (0.1 μg/ml) + 3TC (0.05 μg/ml).
[h] Mutant virus strains that emerged under therapy with MKC-442 (0.04 μg/ml) as single drug or with 3TC (0.02 μg/ml) + MKC-442 (0.02 μg/ml).
[i] Mutant virus strain that emerged under therapy with the combination of MKC-442 (0.04 μg/ml) + 3TC (0.02 μg/ml).

(Lys^{138}, Ile/Val^{184}, or both for TSAO and UC10 or Cys^{181} for MKC-442 and BHAP). These virus mutant strains containing both Lys^{138} and Ile/Val^{184} in their RT remained sensitive to the other NNRTIs, such as BHAP and MKC-442. The latter observations indicate the feasibility of adding another NNRTI to the combinations of 3TC with TSAO-m^3T or thiocarboxanilides to further potentiate the suppressive effect of these combinations on HIV replication. Indeed, we found that the combination of 3TC (0.1 μg/ml) with UC42 (0.04 μg/ml) and the BHAP derivative U90152 (0.04 μg/ml) or the HEPT derivative MKC-442 (0.04 μg/ml), as well as the combination of 3TC (0.1 μg/ml) with TSAO-m^3T (0.4 μg/ml) and MKC-442 (0.04 μg/ml), resulted in complete suppression of virus breakthrough in HIV-1-infected CEM cell cultures at drug concentrations that readily allowed virus replication when single drugs were administered. The molecular basis for the marked suppression or delay of the emergence of resistant virus strains resides in the complementarity of the sensitivity/resistance spectrum of 3TC and the NNRTIs rather than in a potential synergistic activity of 3TC and the NNRTIs against their target enzyme RT. Indeed, it was determined that the combination of 3TC-TP and several NNRTIs (i.e., the thiocarboxanilide UC10, the BHAP derivative U90152, TSAO-m^3T, and MKC-442) exerted an additive inhibitory effect against RT, using poly(I).oligo(dC) as the template/primer (data not shown).

It is somewhat puzzling that the Glu^{138}-to-lysine mutation appeared in the presence of the combination of 0.02 μg/ml 3TC plus 0.05 μg/ml UC10 and the Tyr^{181}-to-cysteine mutation appeared in the presence of the combination of 0.02 μg/ml 3TC plus 0.02 μg/ml MKC-442. Indeed, Glu^{138}-to-lysine and Tyr^{181}-to-cysteine mutant virus strains were previously found to be sensitive to the inhibitory effects of UC10 and 3TC, respectively, at the concentrations used under our experimental conditions (Table 1). However, the most likely explanation may be the markedly higher virus input in the experiments where resistant viruses were selected (Tables 2 and 3) than in those experiments (Table 1) where sensitivity of NNRTIs to different mutant virus strains was evaluated. A higher virus input may lower the antiviral efficacy of the test compounds and thus enable the selection of some mutant virus strains that under normal conditions would be efficiently suppressed. Alternatively, it cannot be excluded that these virus strains may contain additional mutations that are located outside of the sequenced 50–270 amino acid region of the RT and/or that mutations in proteins other than the RT that may play a role in the RT-associated polymerization process.

It should be pointed out that the BHAP derivative U90152 is the subject of extensive clinical trials, whereas the HEPT derivative MKC-442 represents the clinical candidate among the HEPT derivatives. Also, the thiocarboxanilide derivative UC10 is under consideration as a candidate compound for clinical trials in HIV-1-infected patients. In fact, the thiocarboxanilides represent a new group of NNRTIs that are structurally related to the oxathiin carboxanilide UC84 and show a much broader activity spectrum than UC84 against virus mutant strains (27–29). The thiocarboxanilide UC10 has an excellent oral bioavailability profile in rats (70–80%) and dogs (~90%), and it shows strong inhibitory activity against Ala^{106} RT and Cys^{181} RT mutant viruses (27). These properties make UC10 an ideal candidate compound to be included in combinations of 3TC with NNRTIs. The marked potentiation of the antiviral activity of TSAO, the carboxanilide UC10, the BHAP derivative U90152, and the HEPT derivative MKC-442 on combination with 3TC point to the general applicability of the principle of marked antiviral potentiation between 3TC and NNRTIs. It should be mentioned that Larder *et al.* (30) recently reported sustained antiretroviral efficacy of AZT/3TC combination therapy. This particular combination has been the most efficacious pair of drugs tried to date with respect to the magnitude and duration of CD4 cell number changes and viral load. These observations justify extensive clinical studies with paired combinations of 3TC and NNRTIs as well.

From our studies, it is evident that certain drug combina-

tions, such as 3TC with NNRTIs, may be advantageous when given at the highest possible attainable dose *in vivo* to HIV-1-infected individuals. This has not been evident for other drug combinations. Indeed, it has been reported that high-dose therapy of AZT and DDI did not result in better suppression of mutant virus strains than low-dose drug therapy (31). In contrast, high-dose therapy with the NNRTI nevirapine (32) or the HIV protease inhibitor saquinavir (31) led to a sustained antiviral effect. Thus, by combining 3TC with NNRTIs at the highest possible *in vitro* (knock-out) doses, a more efficient and more prolonged suppression of the emergence of drug-resistant virus strains may be achieved *in vivo*. However, the durability of this virus suppression in drug-treated patients remains to be assessed and cannot be predicted from our *in vitro* data. Indeed, AIDS patients have virus titers that are several orders of magnitude higher than the virus titers present in our *in vitro* experiments; they may contain double mutants at the start of the chemotherapy and/or more easily select for double-mutant virus strains starting from single mutants that are present before the chemotherapeutic intervention. However, the latter considerations should not detract from the use of combinations of drugs with a different mechanism of antiviral action such as NNRTIs and 3TC in future therapeutic modalities for HIV-1-infected individuals.

Our results also made it clear that the virus should not be given the opportunity to accumulate resistance mutations in its RT genome. Such sequential accumulation of mutations would make the virus easily resistant to the single and multiple drug combinations (see the double mutated virus strains in Table 3), and this could easily occur when the drug or drugs are applied at concentrations that are too low to efficiently suppress the virus replication and therefore do not prevent virus breakthrough. Full suppression of virus and, thus, prevention of virus breakthrough can be easily achieved *in vitro* with the use of drug combinations (i.e., 3TC with NNRTIs) at concentrations that could be readily attained therapeutically *in vivo*.

In conclusion, our data provide strong evidence that antiviral treatment of HIV-1-infected individuals should be performed not only with the right drug combinations but also at the highest possible drug doses to delay breakthrough of virus and suppress replication of virus, regardless of whether it is drug resistant. Our observations strongly argue for the combination of different HIV inhibitors, such as those presented here (3TC plus NNRTIs), to suppress virus replication and to delay, as long as possible, the emergence of drug-resistant viruses in HIV-1-infected individuals. Our findings indicate that the ddN analogue 3TC, as well as a non-nucleoside RT inhibitor such as BHAP (or other NNRTIs such as nevirapine or α-APA), should be part of such drug combination strategies for the treatment of HIV infections. The thiocarboxanilide derivative UC10 and the HEPT derivative MKC-442 should be further evaluated in preclinical and clinical studies as potential new agents to be used in combination chemotherapy with 3TC.

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References

1. Cooney, D. A., M. Dalal, H. Mitsuya, J. B. McMahon, M. Nadkarni, J. Balzarini, S. Broder, and D. G. Johns. Initial studies on the cellular pharmacology of 2′,3′-dideoxycytidine, an inhibitor of HTLV-III infectivity. *Biochem. Pharmacol.* **35**:2065–2068 (1986).
2. Furman, P. A., J. A. Fyfe, M. H. St. Clair, K. Weinhold, J. L. Rideout, G. A. Freeman, S. Nusinoff Lehrman, D. P. Bolognesi, S. Broder, H. Mitsuya, and D. W. Barry. Phosphorylation of 3′-azido-3′-deoxythymidine and selective interaction of the 5′-triphosphate with human immunodeficiency virus reverse transcriptase. *Proc. Natl. Acad. Sci. USA* **83**:8333–8337 (1986).
3. Ahluwalia, G., D. A. Cooney, H. Mitsuya, A. Fridland, K. P. Flora, Z. Hao, M. Dalal, S. Broder, and D. G. Johns. Initial studies on the cellular pharmacology of 2′,3′-dideoxyinosine, an inhibitor of HIV infectivity. *Biochem. Pharmacol.* **36**:3797–3801 (1987).
4. St. Clair, M. H., C. A. Richards, T. Spector, K. J. Weinhold, W. H. Miller, A. J. Langlois, and P. A. Furman. 3′-Azido-3-deoxythymidine triphosphate as an inhibitor and substrate of purified human immunodeficiency virus reverse transcriptase. *Antimicrob. Agents Chemother.* **31**:1972–1977 (1987).
5. Balzarini, J., P. Herdewijn, and E. De Clercq. Differential patterns of intracellular metabolism of 2′,3′-didehydro-2′,3′-dideoxythymidine and 3′-azido-2′,3′-dideoxythymidine, two potent anti-human immunodeficiency virus compounds. *J. Biol. Chem.* **264**:6127–6133 (1989).
6. Soudeyns, H., X.-J. Yao, Q. Gao, B. Belleau, J.-L. Kraus, N. Nguyen-Ba, B. Spira, and M. A. Wainberg. Anti-human immunodeficiency virus type 1 activity and *in vitro* toxicity of 2′-deoxy-3′-thiacytidine (BCH-189), a novel heterocyclic nucleoside analog. *Antimicrob. Agents Chemother.* **35**:1386–1390 (1991).
7. Coates, J. A. V., N. Cammack, H. J. Jenkinson, I. M. Mutton, B. A. Pearson, R. Storer, J. M. Cameron, and C. R. Penn. The separated enantiomers of 2′-deoxy-3′-thiacytidine (BCH 189) both inhibit human immunodeficiency virus replication *in vitro*. *Antimicrob. Agents Chemother.* **36**:202–205 (1992).
8. Lisignoli, G., A. Facchini, L. Cattini, M. C. G. Monaco, A. Degrassi, and E. Mariani. *In vitro* toxicity of 2′,3′-dideoxy-3′-thiacytidine (BCH189/3TC), a new synthetic anti-HIV-1 nucleoside. *Antiviral Chem. Chemother.* **3**:299–303 (1992).
9. Schinazi, R. F., C. K. Chu, A. Peck, A. McMillan, R. Mathis, D. Cannon, L.-S. Jeong, J. W. Beach, W.-B. Choi, S. Yeola, and D. C. Liotta. Activities of the four optical isomers of 2′,3′-dideoxy-3′-thiacytidine (BCH-189) against human immunodeficiency virus type 1 in human lymphocytes. *Antimicrob. Agents Chemother.* **36**:672–676 (1992).
10. Sommadossi, J.-P., R. F. Schinazi, C. K. Chu, and M.-Y. Xie. Comparison of cytotoxicity of the (−)- and (+)-enantiomer of 2′,3′-dideoxy-3′-thiacytidine in normal human bone marrow progenitor cells. *Biochem. Pharmacol.* **44**:1921–1925 (1992).
11. De Clercq, E. HIV resistance to reverse transcriptase inhibitors. *Biochem. Pharmacol.* **47**:155–169 (1993)
12. Nunberg, J. H., W. A. Schleif, E. J. Boots, J. A. O'Brien, J. C. Quintero, J. M. Hoffman, Jr., E. A. Emini, and M. E. Goldman. Viral resistance to human immunodeficiency virus type 1-specific pyridinone reverse transcriptase inhibitors. *J. Virol.* **65**:4887–4892 (1991).
13. Richman, D., C.-K. Shih, I. Lowy, J. Rose, P. Prodanovich, S. Goff, and J. Griffin. Human immunodeficiency virus type 1 mutants resistant to non-nucleoside inhibitors of reverse transcriptase arise in tissue culture. *Proc. Natl. Acad. Sci. USA* **88**:11241–11245 (1991).
14. Mellors, J. W., G. E. Dutschman, G.-J. Im, E. Tramontano, S. R. Winkler, and Y.-C. Cheng. *In vitro* selection and molecular characterization of human immunodeficiency virus-1 resistant to non-nucleoside inhibitors of reverse transcriptase. *Mol. Pharmacol.* **41**:446–451 (1992).
15. Balzarini, J., A. Karlsson, M.-J. Pérez-Pérez, M.-J. Camarasa, and E. De Clercq. Knocking-out concentrations of HIV-1-specific inhibitors completely suppress HIV-1 infection and prevent the emergence of drug-resistant virus. *Virology* **196**:576–585 (1993).
16. Balzarini, J., A. Karlsson, M.-J. Pérez-Pérez, M.-J. Camarasa, W. G. Tarpley, and E. De Clercq. Treatment of human immunodeficiency virus type 1 (HIV-1)-infected cells with combinations of HIV-1-specific inhibitors results in a different resistance pattern than does treatment with single-drug therapy. *J. Virol.* **67**:5353–5359 (1993).
17. Balzarini, J., A. Karlsson, V. V. Sardana, E. A. Emini, M.-J. Camarasa, and E. De Clercq. Human immunodeficiency virus 1 (HIV-1)-specific reverse transcriptase (RT) inhibitors may suppress the replication of specific drug-resistant (E138K) RT HIV-1 mutants or select for highly resistant (Y181C → C181I) RT HIV-1 mutants. *Proc. Natl. Acad. Sci. USA* **91**:6599–6603 (1994).
18. Balzarini, J., J.-P. Kleim, G. Riess, M.-J. Camarasa, E. De Clercq, and A. Karlsson. Sensitivity of (138 Glu → lysine) mutated human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) to HIV-1-specific RT inhibitors. *Biochem. Biophys. Res. Commun.* **201**:1305–1312 (1994).
19. Gu, Z., Q. Gao, X. Li, M. A. Parniak, and M. A. Wainberg. Novel mutation in the human immunodeficiency virus type 1 reverse transcriptase gene that encodes cross-resistance to 2′,3′-dideoxyinosine and 2′,3′-dideoxycytidine. *J. Virol.* **66**:7128–7135 (1992).

20. Boucher, C. A. B., N. Cammack, P. Schipper, R. Schuurman, P. Rouse, M. A. Wainberg, and J. M. Cameron. High-level resistance to (−) enantiomeric 2′-deoxy-3′-thiacytidine in vitro is due to one amino acid substitution in the catalytic site of human immunodeficiency virus type 1 reverse transcriptase. *Antimicrob. Agents Chemother.* **37**:2231–2234 (1993).
21. Gao, Q., Z. Gu, M. A. Parniak, J. Cameron, N. Cammack, C. Boucher, and M. A. Wainberg. The same mutation that encodes low-level human immunodeficiency virus type 1 resistance to 2′:3′-dideoxyinosine and 2′,3′-dideoxycytidine confers high-level resistance to the (−) enantiomer of 2′,3′-dideoxy-3′-thiacytidine. *Antimicrob. Agents Chemother.* **37**:1390–1392 (1993).
22. Schinazi, R. F., R. M. Lloyd, Jr., M.-H. Nguyen, D. L. Cannon, A. McMillan, N. Ilksoy, C. K. Chu, D. C. Liotta, H. Z. Bazmi, and J. W. Mellors. Characterization of human immunodeficiency viruses resistant to oxathiolane-cytosine nucleosides. *Antimicrob. Agents Chemother.* **37**:875–881 (1993).
23. Tisdale, M., S. D. Kemp, N. R. Parry, and B. A. Larder. Rapid *in vitro* selection of human immunodeficiency virus type 1 resistant to 3′-thiacytidine inhibitors due to a mutation in the YMDD region of reverse transcriptase. *Proc. Natl. Acad. Sci. USA* **90**:5653–5656 (1993).
24. Camarasa, M.-J., M.-J. Pérez-Pérez, A. San-Félix, J. Balzarini, and E. De Clercq. 3′-Spiro nucleosides, a new class of specific human immunodeficiency virus type 1 inhibitors: synthesis and antiviral activity of [2′:5′-bis-O-(tert-butyldimethylsilyl)-β-D-xylo- and -ribofuranose]-3′-spiro-5′-[4′-amino-1′,2′-oxathiole 2′,2′-dioxide] (TSAO) pyrimidine nucleosides. *J. Med. Chem.* **35**:2721–2727 (1992).
25. Pérez-Pérez, M. J., A. San-Félix, J. Balzarini, E. De Clercq, and M. J. Camarasa. TSAO analogues: stereospecific synthesis and anti-HIV-1 activity of 1-[2′,5′-bis-O-(tert-butyldimethyl)-β-D-ribofuranosyl]-3′-spiro-5′-(4′-amino-1′,2′-oxathiole 2′,2′-dioxide) pyrimidine and pyrimidine-modified nucleosides. *J. Med. Chem.* **35**:2988–2995 (1992).
26. Pérez-Pérez, M.-J., A. San-Félix, M.-J. Camarasa, J. Balzarini, and E. De Clercq. Synthesis of [1-[2′,5′-bis-O-(t-butyldimethylsilyl)-β-D-xylo- and β-D-ribofuranosyl]thymine]-3′-spiro-5′-[4′-amino-1′,2′-oxathiole-2′,2′-dioxide] (TSAO): novel types of specific anti-HIV agents. *Tetrahedron Lett.* **33**:3029–3032 (1992).
27. Balzarini, J., M.-J. Pérez-Pérez, S. Vélazquez, A. San-Félix, M.-J. Camarasa, E. De Clercq, and A. Karlsson. Suppression of the breakthrough of human immunodeficiency virus type 1 (HIV-1) in cell culture by thiocarboxanilide derivatives when used individually or in combination with other HIV-1-specific inhibitors (i.e., TSAO derivatives). *Proc. Natl. Acad. Sci. USA* **92**:5470–5474 (1995).
28. Balzarini, J., H. Jonckheere, W. A. Harrison, D. C. Dao, J. Anné, E. De Clercq, and A. Karlsson. Oxathiin carboxanilide derivatives, a class of non-nucleoside HIV-1-specific reverse transcriptase inhibitors (NNRTIs) that are active against mutant HIV-1 strains resistant to other NNRTIs. *Antiviral Chem. Chemother.* **6**:169–178 (1995).
29. Balzarini, J., W. G. Brouwer, E. E. Felauer, E. De Clercq, and A. Karlsson. Activity of various thiocarboxanilide derivatives against wild-type and several mutant human immunodeficiency virus type 1 strains. *Antiviral Res.* **27**:219–236 (1995).
30. Larder, B. A., S. D. Kemp, and P. R. Harrigan. Potential mechanism for sustained antiretroviral efficacy of AZT-3TC combination therapy. *Science (Washington D. C.)* **269**:696–699 (1995).
31. Schapiro, J. M., M. Winters, and T. C. Merigan. Mutational analysis of the saquinavir high dose monotherapy study, in *Abstracts of the Fourth International Workshop on HIV Drug-Resistance*. Sardinia, Italy, 73 (1995).
32. Havlir, D. V., V. A. Johnson, D. B. Hall, P. A. Robinson, C. B. Overbay, and D. D. Richman. Factors determining sustained antiviral response to nevirapine, in *Abstracts of the Fourth International Workshop on HIV Drug-Resistance*. Sardinia, Italy, 22 (1995).

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