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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

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Human immunodeficiency virus type 1 (HIV-1)-infected CEM cells were treated by the HIV-1-specific inhibitors bis-heteroarylpiperazine (BHAP), 4,5,6,7-tetrahydro-5-methylimidazo[4,5,1-jk][1,4]benzodiazepin-2(1H)-one (TIBO) R82913, nevirapine, and the N^3-methylthymine derivative of [2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (TSAO-m^3T), as single agents or in combination, at escalating concentrations. When used individually, the compounds led to the emergence of drug-resistant virus strains within two to five subcultivations. The resulting strains were designated HIV-1/BHAP, HIV-1/TIBO, HIV-1/Nev, and HIV-1/TSAO-m^3T, respectively. The mutant viruses showed the following amino acid substitutions in their reverse transcriptase (RT): Leu-100→Ile for HIV-1/BHAP; Lys-103→Asn for HIV-1/TIBO; Val-106→Ala for HIV-1/Nev; and Glu-138→Lys for HIV-1/TSAO-m^3T. Both the Tyr-181→Cys and Val-106→Ala mutations were found in another mutant emerging following treatment with nevirapine at escalating concentrations. The BHAP-resistant virus remained fully sensitive to the inhibitory effects of nevirapine and TSAO-m^3T, whereas the TSAO-m^3T-resistant virus remained fully sensitive to the inhibitory effects of nevirapine and BHAP. When different pairs of nonnucleoside RT inhibitors (i.e., BHAP plus TSAO-m^3T, nevirapine plus TSAO-m^3T, TIBO plus TSAO-m^3T, nevirapine plus TIBO, and BHAP plus nevirapine) were used, resistant virus emerged as fast as with single-drug therapy. In all cases the Tyr-181→Cys mutation appeared; the virus showed markedly reduced sensitivity to all HIV-1-specific inhibitors but retained sensitivity to 2′,3′-dideoxynucleoside analogs such as zidovudine, ddC, and ddI. Our findings argue against simultaneous combination of two different nonnucleoside RT inhibitors that are unable to inhibit HIV-1 mutant strains containing the Tyr-181→Cys mutation when administered as single drugs.

Several classes of structurally different compounds are inhibitory to the replication of human immunodeficiency virus type 1 (HIV-1) strains but not HIV-2, simian immunodeficiency virus, or other DNA or RNA viruses (2, 8, 9, 11, 20–23, 25, 26–28, 30, 31, 38). The common features of these different compounds are as follows: (i) they are highly potent and selective inhibitors of HIV-1; (ii) they are specifically targeted at HIV-1 reverse transcriptase (RT) and are not inhibitory to cellular DNA polymerases (1, 6–8, 13–15, 19, 20, 27, 30, 31, 38, 40, 41); (iii) they are relatively nontoxic to human cells (2, 8, 9, 11, 20, 22, 27, 30, 31, 34); (iv) their selectivity index in cell culture (ratio of cytotoxic concentration to antivirally effective concentration) is very high (up to 100,000) (1, 8, 9, 20, 27, 30, 31, 38); and (v) they rapidly select for resistant HIV-1 strains in vitro (cell culture) and in vivo (patients) (3–5, 10, 16, 17, 25, 26, 29, 35–37, 39).

The rapid emergence of HIV-1 strains resistant to several HIV-1-specific compounds in vitro {i.e., nevirapine, pyridinone, 4,5,6,7-tetrahydro-5-methylimidazo[4,5,1-jk][1,4]benzodiazepin-2(1H)-one (TIBO) R82913, and [2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (TSAO)} and in vivo (i.e., nevirapine and pyridinone) has become a major concern that may affect the further development of these types of compounds. Resistance to the HIV-1-specific inhibitors stems from single-point mutations in the RT gene, resulting in one or two amino acid substitutions in the RT enzyme. Amino acid changes Leu-100→Ile, Lys-103→Asn, Val-108→Ile, Tyr-181→Cys, and Tyr-188→Cys have been associated with pyridinone resistance (3, 17, 18, 29), Val-106→Ala, Tyr-181→Cys, and Tyr-188→Cys have been associated with nevirapine resistance (3, 24, 26, 37, 41), Glu-138→Lys has been associated with TSAO resistance (3–5, 10), and Pro-236→Leu has been associated with bis-heteroarylpiperazine (BHAP) resistance (16).

Recently, we reported that HIV-1 mutant strains containing different amino acid substitutions in the RT enzyme show differences in sensitivity to HIV-1-specific inhibitors. We found that HIV-1 strains that are resistant to TSAO derivatives (based on the Glu-138→Lys mutation) remain fully sensitive to other HIV-1-specific inhibitors (i.e., TIBO, nevirapine, and pyridinone). Also, HIV-1 strains containing the Leu-100→Ile mutation proved resistant to TIBO derivatives but retained sensitivity to TSAO, nevirapine, and to a lesser extent pyridinone. In contrast, the Tyr-181→Cys mutation leads to a decreased sensitivity to all HIV-1-specific inhibitors (3).

We have now conducted a systematic study of the development of virus drug resistance upon exposure of HIV-1-infected cells to combinations of different HIV-1-specific

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TABLE 1. EC_{50}s of the test compounds for HIV-1(III_B) isolated in the presence of the HIV-1-specific inhibitors TSAO-m^3T, nevirapine, and TIBO R82913 in CEM cells[a]

Compound	EC_{50} (μg/ml) in the presence of increasing concn of:										
	No drug[b]	TSAO-m^3T				Nevirapine				TIBO R82913		
		1[c]	2	3	4	1	2	3	4	1	2	3
TSAO-T	0.03	2	5	2	5	1	2	3	3	0.11	0.12	0.23
TSAO-m^3T	0.03	>50	>50	>50	>50	50	>50	50	>50	0.04	0.03	0.15
TSAO-e^3T	0.03	>50	>50	>50	>50	≥50	>50	≥50	>50	0.06	0.07	0.23
Nevirapine	0.03	0.015	0.003	0.005	0.01	2.6	5	≥5	8.3	0.05	0.08	1.5
Pyridinone	0.007	0.10	0.02	0.05	0.05	2	2	2	3	0.09	0.04	0.5
TIBO R82913	0.02	0.04			0.04	0.4			0.6	0.12	0.20	≥5
BHAP	0.03	0.02			0.03	0.85			8.0	0.02		2

[a] Virus was isolated after full breakthrough of cytopathicity in CEM cell cultures in the presence of TSAO-m^3T at 0.1 μg/ml (passage 1), 0.5 μg/ml (passage 2), 2.5 μg/ml (passage 3), and 10 μg/ml (passage 4); in the presence of nevirapine at 0.04 μg/ml (passage 1), 0.2 μg/ml (passage 2), 2.5 μg/ml (passage 3), and 10 μg/ml (passage 4); and in the presence of TIBO R82913 at 0.01 μg/ml (passage 1), 0.25 μg/ml (passage 2), and 1.0 μg/ml (passage 3).
[b] Values for wild-type HIV-1(III_B) that had not been exposed to any test compound before evaluation for susceptibility to the different HIV-1-specific inhibitors.
[c] Passage number of HIV-1(III_B) cells.

inhibitors. The compounds chosen for constructing the pairs were those that when used as single agents select for mutants with different mutations in the RT enzyme that should retain sensitivity to one another. These investigations were aimed at establishing a treatment modality that prevents the rapid emergence of drug-resistant virus mutants.

MATERIALS AND METHODS

Test compounds. Synthesis of the TSAO derivatives of thymine (TSAO-T), N^3-methylthymine (TSAO-m^3T), and N^3-ethylthymine (TSAO-e^3T) has been described elsewhere (11, 32, 33). TIBO R82913 was kindly provided by Zhang Hao (National Institutes of Health, Bethesda, Md.) or obtained from Pharmatech International Inc. (West Orange, N.J.). Nevirapine (BI-RG-587) and pyridinone L-697,661 were provided by P. Ganong (Boehringer Ingelheim, Ridgefield, Conn.) and M. Goldman (Merck, Sharp & Dohme, West Point, Pa.), respectively. BHAP (U-88204E) was from The Upjohn Company (Kalamazoo, Mich.).

Cells and viruses. CEM cells were obtained from the American Tissue Cell 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 R. C. Gallo and M. Popovic (National Institutes of Health, Bethesda, Md.).

Selection of HIV-1(III_B) mutant strains resistant to HIV-1-specific inhibitors administered as a single drug or in combination. HIV-1(III_B) was subjected to two to three passages in 5-ml CEM cell cultures (4×10^5 cells per ml) in the presence of two to three times the 50% effective concentration (EC_{50}) of TSAO-m^3T (0.1 μg/ml), nevirapine (0.04 μg/ml), TIBO R82913 (0.01 μg/ml), and BHAP (0.1 μg/ml) in 25-cm^2 culture flasks (Falcon; Becton Dickinson) to produce mutant strains designated HIV-1/TSAO-m^3T, HIV-1/Nev, HIV-1/TIBO, and HIV-1/BHAP, respectively. The culture medium consisted of RPMI 1640 containing 10% fetal bovine serum, 2 mM glutamine, and 0.075% $NaHCO_3$. The multiplicity of the initial infection was 200 times the 50% cell culture infective dose. Passages were performed every 3 to 4 days by adding 0.5 to 1.0 ml of the infected culture supernatant to 5 ml of a suspension containing 4×10^5 uninfected CEM cells per ml. As soon as syncytium formation became abundant in the cell cultures, the supernatants were frozen in aliquots at −70°C and the virus was further passaged in the presence of 4-, 5-, or 10-fold higher concentrations of the test compounds. In the drug combination (TSAO-m^3T with nevirapine, TSAO-m^3T with TIBO R82913, TSAO-m^3T with BHAP, nevirapine with BHAP, and TIBO R82913 with BHAP) experiments, the compounds were combined at the same initial concentrations as used for the single-drug treatment; when syncytium formation became abundant in the HIV-1-infected cell cultures, both drug concentrations were increased concomitantly to a 4-, 5-, or 10-fold higher concentration as done for the single-drug treatment procedure.

Sensitivity of several HIV-1 mutant strains to the various test compounds in CEM cells. CEM cells were suspended at 250,000 cells per ml of culture medium and infected with HIV-1(III_B) or the mutant HIV-1 strains at 100 50% cell culture infective doses per ml. 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°C, the cell cultures were examined for syncytium formation. The EC_{50} was determined as the compound concentration required to inhibit syncytium formation by 50%.

Preparation of mutant HIV-1-infected MT-4 cell cultures for polymerase chain reaction analysis and sequencing of the *pol* gene of the mutant HIV-1 strains. The procedure has been described recently (3, 4, 10). Oligonucleotides were chosen to give a 727-bp fragment covering RT amino acids 50 to 270.

RESULTS

Antiviral sensitivity of HIV-1 mutant strains selected for resistance against different HIV-1-specific inhibitors used individually. Virus-induced giant cell formation in the presence of the lowest concentrations of the test compounds appeared after three to five subcultivations. Marked differences were found in the degree of sensitivity of the mutant HIV-1 strains to the HIV-1-specific inhibitors (Tables 1 to 3). TSAO-m^3T-resistant HIV-1 strains remained sensitive to the inhibitory effect of nevirapine and BHAP and showed only slightly diminished sensitivity to TIBO R82913 and pyridinone (Table 1). Also, BHAP-resistant HIV-1 strains remained fully sensitive to the inhibitory effects of nevirapine and TSAO (Table 2).

In contrast, HIV-1 mutant strains selected for resistance against nevirapine proved completely cross-resistant to all other HIV-1-specific inhibitors in one experiment (in which nevirapine was also combined with TSAO-m^3T and TIBO R82913) (Table 1) but remained partially sensitive to pyrid-

TABLE 2. EC_{50}s of the test compounds for HIV-1(III_B) isolated in the presence of the HIV-1-specific inhibitors nevirapine, BHAP, and TSAO-m^3T as single agents or in combination in CEM cells[a]

Compound	EC_{50} (μg/ml)												
	No drug	Nevirapine			BHAP			TSAO-m^3T + BHAP			Nevirapine + BHAP		
		1[b]	2	3	1	2	3	1	2	3	1	2	3
TSAO-T	0.03	0.50	0.77	1.93	0.07	0.06	0.07	1.0	1.4	0.8	0.75	0.63	2.5
TSAO-m^3T	0.03	0.87	0.90	2.5	0.04	0.04	0.04	0.71	0.95	0.44	0.70	0.75	1.4
TSAO-e^3T	0.03	4.3	14	26	0.13	0.13	0.08	1.5	30	8	25	14	50
Nevirapine	0.03	1.2	1.1	2.3	0.04	0.03	0.04	1.9	3.0	2.2	0.75	1.75	4.60
Pyridinone	0.007	0.24	0.08	0.24	0.15	0.12	0.10	5.8	≥10	4.7	5.0	7.0	5.2
TIBO R82913	0.02	0.50	0.63	0.50	0.42	0.41	0.27	0.33	0.37	0.28	0.43	1.1	1.1
BHAP	0.03	0.20	0.20	0.40	0.27	0.63	2.5	1.1	1.3	1.3	1.1	1.1	2.8

[a] Virus was isolated after full breakthrough of cytopathicity in CEM cell cultures in the presence of nevirapine, BHAP, TSAO-m^3T plus BHAP, or nevirapine plus BHAP at 0.1 μg/ml (passage 1), 0.5 μg/ml (passage 2), or 2.5 μg/ml (passage 3).
[b] Passage number of HIV-1(III_B) cells.

inone, TIBO R82913, and BHAP in another experiment (in which nevirapine was also combined with BHAP) (Table 2). The HIV-1 strains that emerged under treatment with escalating concentrations of TIBO R82913 became progressively resistant to TIBO R82913, nevirapine, and BHAP but remained partially sensitive to TSAO derivatives and pyridinone. Also, the virus that was selected for resistance to BHAP became progressively more resistant to BHAP when escalating drug concentrations were used (Table 2).

Antiviral sensitivity of HIV-1 mutant strains selected for resistance against pairs of different HIV-1-specific inhibitors. There was no marked difference in the time of appearance of HIV-1-induced giant cell formation in the cell cultures treated with combinations of the different HIV-1-specific inhibitors compared with the cell cultures treated with the HIV-1-specific inhibitors as single agents. As a rule, paired combination of HIV-1-specific inhibitors resulted in a more pronounced resistance to the individual HIV-1-specific inhibitors than that observed following single-agent treatment. For example, the virus that emerged under TSAO-m^3T–BHAP treatment was more resistant to nevirapine and pyridinone than was the virus that emerged following single-compound (either TSAO-m^3T or BHAP) treatment (Tables 1 and 2). Likewise, the virus that emerged under combined nevirapine-BHAP treatment was more resistant to pyridinone and TIBO R82913 than was the virus that emerged under treatment with either nevirapine or BHAP (Table 2). Also, the combination of TSAO-m^3T with TIBO R82913 resulted in virus that was markedly more resistant to the other HIV-1-specific inhibitors than was virus that had been emerged under treatment by either TSAO-m^3T or TIBO R82913 (Tables 1 and 3).

In virtually all cases, the mutant virus strains that were isolated after combination of compounds at the highest concentrations tested (i.e., TSAO-m^3T [5 μg/ml] with nevirapine (2 μg/ml), TSAO-m^3T [5 μg/ml] with TIBO R82913 [0.5 μg/ml], nevirapine [2.0 μg/ml] with TIBO R82913 [0.5 μg/ml], and nevirapine [2.5 μg/ml] with BHAP [2.5 μg/ml]) proved highly resistant to all HIV-1-specific inhibitors. Only the virus that appeared under the combination of TSAO-m^3T with BHAP retained partial sensitivity to TIBO R82913, TSAO-T, and TSAO-m^3T. The drug susceptibility pattern of the HIV-1 strain that emerged in the presence of TSAO-m^3T plus BHAP did not markedly change when the strain was further subcultured for at least five additional passages in the presence of the combination of the test compounds (data not shown).

Molecular characterization of mutant HIV-1 strains. When the RT genes of the virus isolates emerging under single-compound treatment were sequenced, we found for the TSAO-m^3T-resistant virus a single amino acid mutation from Glu to Lys at position 138, for the BHAP-resistant virus a change from Leu to Ile at position 100, and for the TIBO R82913-resistant virus a change from Lys to Asn at position 103. For the two independently obtained nevirapine-resistant HIV-1 mutant strains, different mutations were demon-

TABLE 3. EC_{50}s of the test compounds for HIV-1(III_B) isolated in the presence of combinations of different HIV-1-specific inhibitors in CEM cells[a]

Compound	EC_{50} (μg/ml)										
	No drug	TSAO-m^3T + nevirapine				TSAO-m^3T + TIBO R82913			Nevirapine + TIBO R82913		
		1[b]	2	3	4	1	2	3	1	2	3
TSAO-T	0.03	0.8	1.5	1.6	5.0	1.5	3.3	3.6	1.2	3.3	3.0
TSAO-m^3T	0.03	1.0	15	≥50	>50	≥50	≥50	>50	0.35	50	>50
TSAO-e^3T	0.03	35	>50	>50	≥50	>50	≥50	>50	10	35	>50
Nevirapine	0.03	1.6	2.5	2.0	>5	1.2	2	≥5	1.5	≥5	>5
Pyridinone	0.007	2.6	≥5	≥5	1.8	1.5	3	2.7	2	≥5	≥5
TIBO R82913	0.015	0.2	1.0		5.0	1.0	0.9	≥5	0.35	1.1	>5

[a] Virus was isolated after full breakthrough of cytopathicity in CEM cell cultures in the combined presence of TSAO-m^3T plus nevirapine at 0.1 plus 0.04 μg/ml (passage 1), 0.25 plus 0.1 μg/ml (passage 2), 1.25 plus 0.5 μg/ml (passage 3), or 5 plus 2 μg/ml (passage 4); in the combined presence of TSAO-m^3T plus TIBO R82913 at 0.1 plus 0.01 μg/ml (passage 1), 1.0 plus 0.1 μg/ml (passage 2), or 5 plus 0.5 μg/ml (passage 3); or in the combined presence of nevirapine plus TIBO R82913 at 0.04 plus 0.01 μg/ml (passage 1), 0.5 plus 0.125 μg/ml (passage 2), or 2.0 plus 0.5 μg/ml (passage 3).
[b] Passage number of HIV-1(III_B) cells.

TABLE 4. Amino acid changes in HIV-1 mutant strains selected for resistance against the HIV-1-specific inhibitors nevirapine, TIBO R82913, and TSAO-m^{3}T used either as single agents or in combination

Amino acid position	HIV-1(III$_B$)	Amino acid in:															
		HIV-1/Nev (expt 1)		HIV-1/TIBO			HIV-1/TSAO-m^{3}T + Nev				HIV-1/TSAO-m^{3}T + TIBO			HIV-1/Nev + TIBO		
		1[a]	4	1	2	3	1	2	3	4	1	2	3	1	2	3
100	Leu (TTA)	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu
103	Lys (AAA)	Lys	Lys	Lys	Asn[b] (AAC)	Asn (AAC)	Lys	Lys	Lys	Lys	Lys	Lys	Lys	Lys	Lys	Lys
106	Val (GTA)	Ala[c] (GCA)	Ala[c] (GCA)	Val	Val	Val	Val	Val	Val	Ala (GCA)	Val	Val	Val	Val	Ala (GCA)	Ala (GCA)
138	Glu (GAG)	Glu	Glu	Glu	Glu	Glu	Glu	Glu	Glu	Glu	Glu	Glu	Glu	Glu	Glu	Glu
181	Tyr (TAT)	Cys[c] (TGT)	Cys[c] (TGT)	Tyr	Tyr	Tyr	Cys (TGT)	Cys (TGT)	Cys (TGT)	Cys (TGT)	Cys (TGT)	Cys (TGT)	Cys (TGT)	Cys[d] (TGT)	Cys (TGT)	Cys (TGT)

[a] Passage number.
[b] Partially wild type (Lys-103) and mutant (Asn-103), with predominance of wild type.
[c] Mixture of Cys-181 (TGT) and Ala-106 (GCA).
[d] Partially wild type (Tyr-181) and mutant (Cys-181).

TABLE 5. Amino acid changes in HIV-1 mutant strains selected for resistance against the HIV-1-specific inhibitors TSAO-m^{3}T, nevirapine, and BHAP used either as single agents or in combination

Amino acid position	HIV-1(III$_B$)	Amino acid in:														
		HIV-1/TSAO-m^{3}T		HIV-1/Nev (expt 2)			HIV-1/BHAP			HIV-1/TSAO-m^{3}T + BHAP			HIV-1/Nev + BHAP		
		1[a]	4	1	2	3	1	2	3	1	2	3	1	2	3
100	Leu (TTA)	Leu	Leu	Leu	Leu	Leu	Ile[b] (ATA)	Ile (ATA)	Ile (ATA)	Leu	Leu	Leu	Leu	Leu	Leu
103	Lys (AAA)	Lys	Lys	Lys	Lys	Lys	Lys	Lys	Lys	Lys	Lys	Lys	Lys	Lys	Lys
106	Val (GTA)	Val	Val	Ala[c] (GCA)	Ala (GCA)	Ala (GCA)	Val	Val	Val	Val	Val	Val	Val	Val	Ala[c] (GCA)
138	Glu (GAG)	Lys (AAG)	Lys (AAG)	Glu	Glu	Glu	Glu	Glu	Glu	Glu	Glu	Glu	Glu	Glu	Glu
181	Tyr (TAT)	Tyr	Tyr	Tyr	Tyr	Tyr	Tyr	Tyr	Tyr	Cys (TGT)	Cys (TGT)	Cys (TGT)	Cys (TGT)	Cys (TGT)	Cys (TGT)

[a] Passage number.
[b] Partially Ile-100 (ATA) and Met-100 (ATG).
[c] Partially wild-type Val-106 and mutant Ala-106.

strated. For the HIV-1 strains that emerged under treatment with escalating nevirapine concentrations in the experiment in which the combination of nevirapine with TSAO-m^{3}T was evaluated (Table 4), a mixture of the Val→Ala mutation at position 106 and the Tyr→Cys mutation at position 181 was observed. However, in the experiment in which nevirapine was combined with BHAP or TIBO 82913 (Table 5), only the Val-106→Ala mutation was noted.

All virus strains that emerged following combination therapy invariably had the Tyr→Cys mutation at position 181 (Tables 4 and 5). In addition to the Tyr-181→Cys mutation, the Val-106→Ala mutation appeared at the highest drug concentrations used, i.e., for strains treated with TSAO-m^{3}T plus nevirapine, TSAO-m^{3}T plus TIBO R82913, nevirapine plus BHAP, and nevirapine plus TIBO R82913. It is paradoxical that the Tyr-181→Cys mutation did not arise following treatment of HIV-1-infected cultures with HIV-1-specific inhibitors other than nevirapine but did arise following paired combinations of HIV-1-specific inhibitors not including nevirapine.

DISCUSSION

Among the different classes of HIV-1-specific inhibitors, only TSAO and BHAP derivatives consistently select for HIV-1 mutant strains that contain amino acid changes in their RT which are located in a region other than the 179-to-190 region. This has now been confirmed for nine different TSAO-resistant HIV-1 strains (3, 4, 10) and five different BHAP (U-87204, U-88204, and U-90152)-resistant HIV-1 strains (16, 39; this report and data not shown). Moreover, each of both types of drug-resistant HIV-1 strains remained fully sensitive to the other class of compounds. In contrast, the HIV-1 mutant strains that contain a mutation in the 179-to-190 region (i.e., Tyr→Cys at position 181) lose, to a variable extent, their sensitivity to all classes of HIV-1-specific inhibitors, including TSAO and BHAP.

As demonstrated by our present findings, the amino acid 181 mutation invariably appeared regardless of the pair of compounds combined, including TSAO-m^{3}T plus BHAP, which usually select for mutations outside the 179-to-190 region when used as single drugs. Whereas the HIV-1-specific inhibitors nevirapine and TIBO R82913, if used as single agents, selected for a mutation different from the Tyr-181→Cys mutation (i.e., Val-106→Ala for nevirapine and Lys-103→Asn for TIBO R82913), combination of these compounds with each other or with one of the other compounds (i.e., TSAO-m^{3}T or BHAP) consistently resulted in the appearance of the Tyr-181→Cys mutation. It should be mentioned that nevirapine and TIBO R82913 may also select for resistant viruses that contain mutations other than those observed under our experimental conditions (3, 26, 36, 37).

As TSAO-m^{3}T and BHAP show a complete lack of cross-resistance, it may have been expected that when the two were combined, BHAP would suppress the emergence of the TSAO-m^{3}T-specific mutation at position 138 (Glu→Lys) and vice versa, TSAO-m^{3}T would suppress the emergence of the BHAP-specific mutation at position 100 (Leu→Ile). Unexpectedly, however, the combination of TSAO-m^{3}T and BHAP led to selection of the Tyr→Cys mutation at position 181, although neither compound did so when used individually.

Interestingly, the Leu-100→Ile mutation occurred in BHAP (U-88204)-resistant HIV-1 strains. This result is in agreement with the findings of Vasudevachari and coworkers (39) using BHAP U-88204 as the selecting agent but contrasts with a recent report by Dueweke and coworkers (16), who found a Pro-236→Leu mutation in the RT of BHAP (U-87201 and U-90152)-resistant viruses. Moreover, HIV-1 strains containing the Pro-236→Leu mutation were shown to be more sensitive to the inhibitory effects of nevirapine, TIBO, and pyridinone than was the wild-type virus (16). Such increased sensitivity to other HIV-1-specific inhibitors was not found for the Leu-100→Ile HIV-1 mutant strains emerging under our experimental conditions. Also, a TIBO-resistant HIV-1 strain containing the Leu-100→Ile mutation did not confer higher sensitivity to other HIV-1-specific inhibitors (4). However, recently we noted the appearance of a Pro-236→Leu mutation in the passage 3 HIV-1/BHAP strain) (Table 5) that was further selected for superresistance in the presence of 10 μg of BHAP per ml. Thus, in addition to the Leu-100→Ile mutation, this BHAP-superresistant HIV-1 strain acquired the Pro-236→Leu mutation (data not shown). These observations confirm the importance of the Pro-236→Leu mutation for BHAP and also indicate that differences in the selection process may result in different mutations.

It is puzzling that TSAO and BHAP did not select for the Tyr-181→Cys mutation when used as single agents but selected for this mutation only when combined. Assuming that the Tyr-181→Cys mutation exists in the HIV-1 population before treatment is started, the fact that it is selected only after combined treatment of TSAO and BHAP may indicate that the Tyr-181→Cys mutant is less pathogenic than the mutants that are specific for either compound used alone (i.e., Glu-138→Lys and Leu-100→Ile mutants).

In several cases, a second mutation occurred only following treatment with escalating concentrations. Most likely, the second mutation is required for the virus to acquire increased resistance to the compounds. These observations argue against an escalating dose treatment regimen with the HIV-1-specific inhibitors and instead favor treatment from the start with the highest possible (loading) doses. Recent data (not shown) from our laboratory indicate that if the HIV-1-infected cell cultures are exposed to sufficiently high concentrations (i.e., 2.5 to 10 μg/ml) of either nevirapine, BHAP, TIBO, or pyridinone, development of virus drug resistance is abrogated and consequently the cells are completely cleared (sterilized) of the virus. Since one of the characteristics of the HIV-1-specific inhibitors (i.e., TIBO, nevirapine, and BHAP) is their extremely low toxicity profile, as revealed by phase I/II studies in patients (34, 36), high-dose therapy of HIV-1-infected individuals with HIV-1-specific inhibitors may be a feasible and achievable goal. For example, plasma levels higher than 10 μg/ml can be easily obtained in BHAP-treated individuals (38a).

Our observations argue against the use of pairs of HIV-1-specific nonnucleoside RT inhibitors, such as used in our present experiments, to delay or prevent emergence of drug-resistant virus. However, Emini and coworkers reported that in pyridinone-treated AIDS patients, emergence of resistant HIV-1 strains containing the Tyr-181→Cys mutation is suppressed by simultaneous zidovudine (AZT) therapy (18). This finding opens interesting perspectives for a combination of AZT with HIV-1-specific inhibitor pairs that together lead to the Tyr-181→Cys mutation. Also, combination of HIV-1-specific inhibitor pairs such as reported in the present study with a nonnucleoside RT inhibitor that shows a marked inhibitory effect on mutant HIV-1 strains containing the Tyr-181→Cys mutation should be pursued as a strategy to circumvent the resistance problem.

Combinations between nucleoside RT inhibitors and non-

nucleoside RT inhibitors may select for replication-defective virus strains, as recently suggested by Chow et al. (12) for the triple combination of AZT, ddI, and nevirapine (or pyridinone). However, it has not been proven that the apparent sterilization of HIV-1-infected cell cultures by such a triple combination of test compounds is based on the accumulation of amino acid mutations that are incompatible with a functional RT enzyme. Moreover, Kellam and Larder (21) reported that HIV-1 can acquire coresistance to AZT, ddI, and nevirapine, and these findings contradict those of Chow et al. (12).

In conclusion, from our data it appears that combination of two different HIV-1-specific nonnucleoside inhibitors for the treatment of HIV-1 does not prevent the development of virus drug resistance. This resistance is invariably based on the Tyr-181→Cys mutation and emerges as rapidly as with single-drug therapy. Furthermore, the Cys-181 mutant exhibits a broader cross-resistance pattern than do the HIV-1 mutants (i.e., Ile-100, Ala-106, or Lys-138) emerging under single-drug therapy. Combination of HIV-1-specific inhibitor pairs with either AZT or other nonnucleoside RT inhibitors that are able to suppress HIV-1 strains containing the Tyr-181→Cys mutation in their RT should be further explored as therapeutic modalities to prevent emergence of virus drug resistance.

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