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Generation of Drug-Resistant Variants of Human Immunodeficiency Virus Type 1 by In Vitro Passage in Increasing Concentrations of 2′,3′-Dideoxycytidine and 2′,3′-Dideoxy-3′-Thiacytidine

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We selected in vitro human immunodeficiency virus type 1 variants that are resistant to each of 2′,3′-dideoxycytidine (ddC) and the racemic mixture of 2′,3′-dideoxy-3′-thiacytidine (BCH-189). The median effective concentrations of ddC and BCH-189 obtained for the resistant viruses ranged between 10 and 50 times above those for parental wild-type strains, and extensive cross-resistance was observed against 2′,3′-dideoxyinosine (ddI) but not 3′-azido-3′-deoxythymidine (AZT). Two dimer compounds, in which either AZT and ddI or AZT and BCH-189 were linked through phosphodiester linkages, did not permit the emergence of variants resistant to BCH-189, ddI, or AZT but were ineffective at inhibiting the replication of AZT-resistant viruses.

The clinical relevance of human immunodeficiency virus (HIV) resistance to 3′-azido-3′-deoxythymidine (AZT) and other antiviral agents is an area of active study (1, 8, 11). We and other investigators have demonstrated the emergence, under cell culture conditions, of HIV type 1 (HIV-1) variants that display resistance to each of AZT and 2′,3′-dideoxyinosine (ddI) (5, 7). It has been reported as well that HIV-1 resistance against nonnucleoside inhibitors of reverse transcriptase (RT) can be selected for under conditions of in vitro viral replication (10).

Because it is apparent that other members of the nucleoside family may be used in the therapy of HIV-associated disease, it is important to determine whether variants of HIV-1 that show diminished resistance to other compounds, such as 2′,3′-dideoxycytidine (ddC) and the (−) enantiomer of 2′,3′-dideoxy-3′-thiacytidine (3TC) (3, 4, 13), will be likely to emerge as well. The racemic mixture from which 3TC is derived, known as BCH-189, is a cytosine analog that possesses anti-HIV activity in both the (+) and the (−) enantiomeric forms (3, 15). We report here that HIV-1 resistance to each of ddC and BCH-189 may be selected for in cell culture as efficiently as has been previously shown for AZT and ddI (5). However, the use of dimer compounds in which either AZT and ddI [3′-azido-3′-deoxythymidilyl-(5′,5′)-2′,3′-dideoxy-5′-inosine; AZT-P-ddI] or AZT and BCH-189 [3′-azido-3′-deoxythymidilyl-(5′,5′)-2′,3′-dideoxy-3′-thiacytidine; AZT-P-BCH-189] were linked through phosphodiester linkages (14) did not permit the development of drug resistance in our in vitro assay, even though these drugs could not efficiently block the replication of AZT-resistant variants of HIV-1.

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Cells and viruses. The MT-4 line of $CD4^+$ lymphocytes was used in the experiments described here. These cells were maintained as suspension cultures (3×10^5 to 5×10^5 cells per ml) in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, and antibiotics as described previously (5). Robert Gallo (National Institutes of Health, Bethesda, Md.) kindly provided the HIV-III_B laboratory strain of HIV-1. In addition, we worked with a number of clinical isolates which were obtained by coculture of peripheral blood lymphocytes from infected individuals with cord blood lymphocytes provided by our hospital's Department of Obstetrics as described previously (11). In particular, we extensively studied HIV-1 isolates from patients 174 and 278; these viruses were obtained at times both prior to and 12 months following initiation of AZT therapy (500 mg/day). Viruses which were recovered were propagated on MT-4 cells as described previously (5) and were stored at −70°C.

AZT, ddI, and ddC were obtained from Wellcome Inc. (Research Triangle Park, N.C.), Bristol-Myers Squibb (Wallingford, Conn.), and Sigma Chemical Co. (St. Louis, Mo.), respectively. BCH-189 was a gift from BioChem Pharma Inc., Montreal, Quebec, Canada. AZT-P-ddI and AZT–P–BCH-189 dimer compounds were gifts from IVAX Corp., Miami, Fla.

Selection of drug-resistant variants of HIV-1. MT-4 cells were preincubated for 30 min with subeffective concentrations of drug and were subsequently infected with either clinical isolates or the HIV-III_B laboratory strain at a multiplicity of infection of 0.01. Virus was first titrated by plaque assay on MT-4 cells as described elsewhere (5). After 3 h, the cells were washed and maintained in culture medium containing the same subeffective concentration of drug used during preincubation. The medium was changed twice weekly, and at each replacement the medium contained a gradually increasing drug concentration, according to the schedule described in Table 1. Culture fluids (0.5 ml) from each round of HIV-1 infection were used to infect fresh MT-4 cells each time as described previously (5). Cells were monitored for the presence of viral p24 antigen and RT

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TABLE 1. Procedure used to select drug-resistant variants of HIV-1

Cycle of infection	Duration of infection cycle (days)	Drug concn (μM)			
		AZT	ddI	ddC	BCH-189
1	6	0.018	19	0.75	0.75
2	6	0.0225	34	1.1	1.1
3	6	0.0325	85	5.2	5.2
4	6	0.0375	169.5	11.5	11.5
5	7	0.75	254	16.4	16.4
6	7	1.5	339	25	25
7	7	1.9	424		
8	7	2.5	655		

activity by indirect immunofluorescence assay and enzyme assay, respectively, as described previously (2, 12). In addition, viral p24 antigen was monitored by an antigen capture assay by using commercially available kits purchased from Abbott Laboratories (North Chicago, Ill.).

The results presented in Table 2 show that significant levels of viral RT activity and p24 antigen could be generated in cell culture as soon as 2 weeks after initial selection for resistance in the presence of AZT. Similar results were obtained with each of ddI, ddC, and BCH-189. After 4 weeks of augmentation of the drug concentration, we were often able to grow drug-resistant variants of each of two clinical isolates (from patients 174 and 278) and the HIV-III_B laboratory strain in concentrations of AZT as high as 2.5 μM, concentrations of ddI as high as 655 μM, and 25 μM (each) ddC and BCH-189. These values are in excess of 50-fold the usual inhibitory concentrations of AZT and 10- to 30-fold those of ddI, ddC, and BCH-189.

Use of AZT dimer compounds. Dimer compounds, in which AZT and other nucleosides were linked through phosphodiester linkages, have been reported to inhibit HIV-1 replication (14). The results in Table 2 indicate that viruses resistant to either AZT or ddI could not be detected after 8 weeks in culture in the presence of either AZT-P-ddI or AZT–P–BCH-189 by procedures identical to those described above for selection of drug resistance to the parental nucleosides.

Table 3 contains summary data on the ability of each of AZT, ddI, ddC, and BCH-189 to inhibit the replication of both wild-type HIV-1 as well as variants that were selected to display resistance against each of these compounds. The median effective concentrations (EC_{50}s) of the various drugs studied were calculated by curve-fitting analysis on the basis of three independent determinations of RT activities in culture fluids as described previously (6) by using viruses that were passaged in MT-4 cells about eight times. Viruses that were resistant to AZT were nonetheless susceptible to each of ddI, ddC, and BCH-189 when they were studied in MT-4 cells. Similarly, viruses selected for resistance against ddI, ddC, and BCH-189 all retained their susceptibilities to AZT. In contrast, however, extensive cross-resistance was

TABLE 2. Generation of drug-resistant variants of HIV-1 by passage in MT-4 cells in the presence of nucleoside analogs

Virus	Drug (initial concn [μM])[a]	RT activity (cpm/ml [10^4]) at the following time after infection[b]:			% Fluorescent cells at the following time after infection[c]:		
		2 wk	4 wk	6 wk	2 wk	4 wk	6 wk
Clinical isolate from patient 174	None	23.5	26.0	29.2	88	82	85
	AZT (0.018)	4.2	17.5	25.7	28	55	79
	ddI (19)	9.3	21.6	27.8	30	46	58
	ddC (0.75)	8.2	20.3	29.5	28	53	76
	BCH-189 (0.75)	7.8	23.5	27.2	32	48	79
Clinical isolate from patient 278	None	19.4	29.2	26.8	91	84	86
	AZT (0.018)	3.8	19.7	27.5	19	63	70
	ddI (19)	10.8	18.4	29.3	28	52	75
	ddC (0.75)	6.3	14.2	24.0	18	51	69
	BCH-189 (0.75)	9.0	15.6	27.4	24	59	62
HIV-III_B	None	17.3	29.8	27.1	79	94	88
	AZT (0.018)	3.9	16.2	29.3	35	62	84
	ddI (19)	8.1	23.5	33.1	28	49	91
	ddC (0.75)	5.6	19.4	27.6	19	61	80
	BCH-189 (0.75)	6.3	22.5	31.7	23	58	89
Clinical isolate from patient 174	None	27.6	19.8	30.8	71	85	82
	AZT (0.018)	5.1	20.3	26.4	19	61	75
	ddI (19)	3.4	16.2	28.0	20	49	83
	AZT-P-ddI (0.018)	1.6	1.4	1.9	0	0	0
	AZT-P-BCH-189 (0.018)	3.5	1.7	2.5	0	0	0
HIV-III_B	None	19.7	18.5	23.8	85	92	88
	AZT (0.018)	2.3	16.7	27.2	22	52	73
	ddI (19)	1.7	12.8	25.6	18	38	67
	AZT-P-ddI (0.018)	2.5	3.4	2.1	0	0	0
	AZT-P-BCH-189 (0.018)	2.8	1.7	3.0	0	0	0

[a] All cultures were maintained at the original concentration of drug for at least 6 days. In cases in which cultures became positive, higher drug concentrations were used, following the schedule described in Table 1.

[b] Data are means of three replicate samples.

[c] Data are means of three replicate samples.

TABLE 3. Effect of nucleoside analogs and dimer compounds on replication of drug-resistant variants of HIV-1

Selection pressure	Origin of virus used	EC_{50} (μM)[a]					
		AZT	ddI	ddC	BCH-189	AZT-P-ddI	AZT-P-BCH-189
None	$HIV\text{-}III_B$	0.03 ± 0.002	4.2 ± 0.5	0.45 ± 0.08	0.55 ± 0.7	ND[b]	ND
None	Patient 263	0.02 ± 0.001	3.6 ± 0.4	0.35 ± 0.04	0.65 ± 0.3	ND	ND
None	$HIV\text{-}III_B$	0.04 ± 0.002	10.6 ± 0.9	0.65 ± 0.08	ND	0.08 ± 0.009	0.05 ± 0.007
None	$HIV\text{-}III_B$	0.06 ± 0.008	10.4 ± 1.2	0.48 ± 0.02	ND	0.02 ± 0.001	0.10 ± 0.008
AZT	$HIV\text{-}III_B$	1.6 ± 0.008	3.9 ± 0.5	0.40 ± 0.06	0.50 ± 0.05	ND	ND
AZT	Patient 263	2.5 ± 0.02	5.2 ± 0.3	0.45 ± 0.05	0.65 ± 0.04	ND	ND
AZT	$HIV\text{-}III_B$	2.2 ± 0.03	10.6 ± 1.4	0.35 ± 0.02	ND	0.95 ± 0.06	0.65 ± 0.05
AZT	$HIV\text{-}III_B$	1.3 ± 0.01	10.5 ± 1.8	0.73 ± 0.11	ND	0.85 ± 0.03	0.31 ± 0.04
ddI	$HIV\text{-}III_B$	0.02 ± 0.003	54.5 ± 3.2	5.2 ± 0.05	6.1 ± 0.4	ND	ND
ddI	Patient 263	0.05 ± 0.002	47.8 ± 6.7	4.3 ± 0.6	4.9 ± 0.5	ND	ND
ddI	$HIV\text{-}III_B$	0.08 ± 0.003	236.4 ± 19.0	0.78 ± 0.05	ND	0.08 ± 0.002	0.05 ± 0.004
ddI	$HIV\text{-}III_B$	0.06 ± 0.004	134.9 ± 12.8	0.55 ± 0.03	ND	0.06	0.03 ± 0.005
ddC	$HIV\text{-}III_B$	0.04 ± 0.001	25.3 ± 3.2	3.2 ± 0.4	6.8 ± 0.4	ND	ND
ddC	Patient 263	0.07 ± 0.004	15.8 ± 2.1	4.8 ± 0.6	10.4 ± 1.3	ND	ND
ddC	$HIV\text{-}III_B$	0.08 ± 0.010	10.76 ± 1.6	8.2 ± 0.6	ND	0.07 ± 0.006	0.06 ± 0.009
BCH-189	$HIV\text{-}III_B$	0.05 ± 0.002	3.7 ± 0.7	5.9 ± 0.3	7.2 ± 0.7	ND	ND
BCH-189	Patient 263	0.02 ± 0.003	16.0 ± 1.1	7.5 ± 0.5	9.0 ± 0.5	ND	ND

[a] Data are means ± standard deviations for three replicate samples.
[b] ND, not done.

documented among viruses selected with each of ddI, ddC, and BCH-189. Interestingly, viruses selected in the presence of increasing concentrations of ddC displayed much higher levels of resistance against BCH-189 than against ddC itself. The results in Table 3 also indicate that AZT-resistant variants of HIV-1 were able to replicate efficiently in the presence of normally inhibitory concentrations of either of the two dimers AZT-P-ddI and AZT–P–BCH-189. In contrast, variants of HIV-1 that were resistant to ddI were not able to replicate in the presence of either dimer.

The major findings presented here relate to our ability to have generated strains of HIV-1 resistant to each of ddC and BCH-189 through in vitro culture and drug pressure procedures. Such resistance against individual nucleosides can develop in culture over periods ranging between 2 and 8 weeks. In our studies, the AZT-resistant variants of HIV-1 that were selected for in culture possessed genotypes similar to those of HIV-1 variants that have been isolated from patients under prolonged therapy with AZT (5). However, this does not necessarily prove that the mechanisms responsible are the same, although other investigators (9) have noted that the same order of *pol* gene mutations is likely to occur both in vivo and in vitro.

What is the basis for the different results obtained when the dimer compounds were used to inhibit replication of drug-resistant variants of HIV-1? Each component of the dimers was present at equimolar concentrations. The dimers are probably active only following intracellular hydrolysis to individual nucleosides, which are then phosphorylated through the usual pathways to yield products with direct antiviral activities. The AZT representation in the dimers is sufficient to impede replication of wild-type HIV-1 isolates when these compounds are present at concentrations of at least 0.1 μM, since the usual EC_{50} of AZT for HIV-1 in MT-4 cells ranges between 0.01 and 0.1 μM. Accordingly, it was not surprising that the dimers were able to impede the replication of ddI-resistant viruses, even when the dimers were used at concentrations as low as 0.05 μM, since a sufficient level of AZT was present to affect those variants which retained an AZT-susceptible phenotype.

In contrast, the levels of ddI and BCH-189 necessary to impede HIV-1 replication were much higher, i.e., 2 to 5 μM. Thus, when used at low concentrations, i.e., 0.1 μM, all of the antiviral activity of these dimers is attributable to the AZT component rather than to the ddI or the BCH-189 component. In the case of variants resistant to AZT, there was insufficient ddI or BCH-189 in the dimers, when the dimers were used at 0.1 to 1 μM, to have an impact on viral replication. Understandably, these compounds were able to impede replication of AZT-resistant variants when the compounds were used at concentrations within the usual median inhibitory concentration range, i.e., 2 to 5 μM, for ddI and BCH-189. Although the relatively low concentrations of ddI and BCH-189 present in the AZT dimers were presumably not able to antagonize replication of AZT-resistant variants of HIV-1, they might nonetheless have acted synergistically with AZT to prevent the emergence of resistance against either AZT or ddI (Table 2).

Cross-resistance to ddI and ddC has been described previously (6, 16). It is therefore not surprising that this should extend as well to BCH-189, which is structurally similar to ddC. It will be of interest to determine whether resistance against 3TC will develop as rapidly in MT-4 cell cultures as that seen for BCH-189.

Finally, we succeeded in selecting for HIV-1 drug resistance by in vitro passage only when we used the MT-4 cell line. Similar attempts which used either human cord blood lymphocytes or human peripheral blood mononuclear cells ended in failure. Clinical trials with the drugs described here will be necessary to determine whether drug resistance will also emerge under conditions of prolonged antiviral chemotherapy.

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