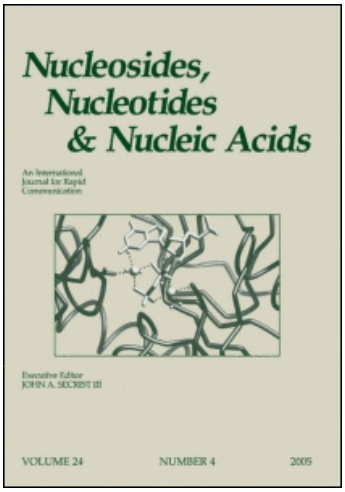

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Resistance Profiles of ( ) 2'-Deoxy-3'-oxa-4'-thiocytidine and (-) 2'-Deoxy-3'-oxa-4'-thio-5-fluorocytidine

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RESISTANCE PROFILES OF (+) 2'-DEOXY-3' -OXA-4' -THIOCYTIDINE AND (-) 2 '-DEOXY-3' -OXA-4' -THIO-5- FLUOROCYTIDINE.

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ABSTRACT. Resistant variants were selected *in vitro* against two novel nucleoside analogues, (+) dOTC and (-) dOTFC using the HIV-1 molecular clone HXB2D. The variants obtained displayed 6.5-fold and 10-fold resistance to these compounds, respectively. Cloning and sequencing of the RT genes of the selected viruses identified two mutations, M184I for (+) dOTC and M184V for (-) dOTFC. Results with mutated recombinant clones of HXB2D confirmed the importance of these mutations in MT-4 cells. The resistance profiles of clinical samples with wild-type or 3TC-resistant phenotypes were also studied; low to moderate levels of cross-resistance were observed against the novel compounds.

INTRODUCTION

The reverse transcriptase (RT) of the human immunodeficiency virus type 1 (HIV-1) is an important target for antiviral chemotherapy since it performs a critical function in viral replication and has no cellular homologue. Nucleoside analogues competitively inhibit reverse transcription by competing with native (dNTPs), and cause chain termination when incorporated into nascent proviral DNA. However, resistant HIV-1 variants have been isolated in patients undergoing prolonged drug therapy and can also be selected *in vitro* [1,2,3].

We have previously shown that four novel cytidine analogues possess antiviral activity *in vitro* [4,5]. These compounds are the (+) and (-) enantiomers of 2 '-deoxy-3' -oxa-4' -thiocytidine (dOTC) and their fluorinated derivatives, i.e. (+) and (-) 2 '-deoxy-3' -oxa-4' -thio-5- fluorocytidine (dOTFC). These compounds are structurally related to

determine whether resistant variants to (+) dOTC and (-) dOTFC could be selected *in vitro* and whether the mutation patterns of such resistant viruses would be similar to that seen with 3TC. Furthermore, we wished to assess the degree of cross-resistance of the selected variants to all the novel compounds in order to evaluate potential for therapeutic utility.

MATERIALS AND METHODS

Viruses and Cells. The infectious molecular clone of HIV-1, HXB2D, was utilized in the selection of resistant variants. Clinical HIV-1 isolates were obtained by co-culturing peripheral blood mononuclear cells (PBMC) from patients with cord blood mononuclear cells (CBMC) as described[6]. The human T cell line MT-4 was utilized to grow both wild type and resistant variants as described[6].

Selection of drug -resistant variants. Resistant variants were selected by growing HIV HXB2D in MT-4 cells. Initially we used a drug concentration of 1μM. This was gradually increased to 200μM over 12 passages. In order to demonstrate selection of resistant variants, the IC_{50}s of the selected viruses were determined and compared to those of wild type virus.

Cloning and Sequencing. Cellular DNA was harvested from MT-4 cells infected with selected variants and the RT genes were amplified by polymerase chain reaction (PCR) using specific primers and cloned and sequenced as described[7]. Once putative mutations were identified recombinant molecular clones of HXB2D containing theses mutations were generated by site-directed mutagenesis[7]. Viral stocks of the mutated recombinant clones were produced in MT-4 cells and utilized to confirm whether the mutations conferred resistance to the compounds used for selection.

Clinical Isolates. The resistance patterns of clinical isolates to the novel compounds were determined in CBMCs in order to evaluate the degree of cross-resistance in patient samples. The clinical isolates used displayed either wild-type or 3TC-resistant phenotypes.

RESULTS

We first sought to determine whether resistant variants could be selected with the compounds (+) dOTC and (-) dOTFC using the wild-type recombinant molecular clone

TABLE 1. Selection of HIV HXB2D variants with (+) dOTC and (-) dOTFC in MT-4 cells.

Drug	IC_{50} (μM)[a]		Mutations identified[b]	No. of passages
	HXB2D	HXB2D-selected		
(+) dOTC	2.0 ± 0.40	13 ± 1.0	M184I	12
(-) dOTFC	3.0 ± 0.41	30 ± 3.0	M184V	12

[a] Results were determined on the basis of RT activity in culture fluids as described[8].

[b] Mutations were identified by cloning and sequencing the RT genes of drug-selected variants as described in Materials and Methods.

HXB2D. During 12 passages *in vitro,* the concentration of drug had gradually been increased from 1μM to 200μM over three months. The IC_{50} values of the selected variants were determined in MT-4 cells. The results demonstrate that increases of 6.5 -fold and 10-fold were observed in the IC_{50} values for (+) dOTC and (-) dOTFC respectively, as shown in Table 1.

In order to elucidate the mechanism of resistance of the selected variants, cloning and sequencing of the RT genes of the selected viruses were performed. The complete sequences of five RT genes were analyzed for each variant, and mutations at position 184, i.e. M184I and M184V, were identified in the clones of (+) dOTC and (-) dOTFC -selected variants, respectively, as illustrated in Table 1. These mutations were then introduced into the molecular clone HXB2D in order to confirm that they conferred resistance to the compounds. The results of Table 2 demonstrate that HXB2D-M184I conferred approximately 5-fold resistance to (+) dOTC and that HXB2D-M184V conferred >15-fold resistance to (-) dOTFC in MT-4 cells. The patterns of cross -resistance of both HXB2D-M184I and M184V to other compounds were also evaluated and are summarized in Table 2. HXB2D-M184I conferred between 4-5-fold resistance to each drug while HXB2D-M184V conferred >10-fold resistance. The M184I and M184V mutations have been previously characterized as conferring low-level and high-level resistance to 3TC, respectively[1,3].

The results described above were obtained using the human MT-4 cell line and a molecular clone of HIV-1. Therefore, it was of interest to also study resistance patterns in primary cells. Clinical isolates which displayed wild-type or 3TC-resistant phenotypes

TABLE 2. Susceptibility of recombinant HXB2D to nucleoside analogues in MT-4 cells.

Drug	IC_{50} (μM)[a]		
	HXB2D	HXB2D-M184I	HXB2D-M184V
(-) dOTC	3.5 ± 0.9	17 ± 2.8	35 ± 6.5
(+) dOTC	3.0 ± 1.0	16 ± 2.0	>100
(-) dOTFC	4.8 ± 1.1	18 ± 3.0	65 ± 15
(+) dOTFC	6.6 ± 1.6	27 ± 5.1	>100
3TC	0.30 ± 0.09	97 ± 4.7	>200

[a] Results were determined on the basis of RT activity in culture fluids as described[8]. Data are means ± standard deviations from three independent experiments.

TABLE 3. Susceptibility of HIV-1 clinical isolates to nucleoside analogues in CBMCs.

Drug	IC_{50} (μM)[a]			
	Wild -type isolates		Isolates resistant to 3TC	
	4242	4246	3887	4205
(-) dOTC	0.20	0.20	0.40	2.5
(+) dOTC	0.25	1.5	1.5	15
(-) dOTFC	0.25	0.30	0.30	15
(+) dOTFC	0.40	0.40	4.0	>100
3TC	0.20	0.025	10	>100
AZT	0.007	0.008	0.002	0.007

[a] Results were determined on the basis of RT activity in culture fluids of CBMCs as described[8]. Data are the average of duplicate results.

were obtained and their susceptibilities to the compounds were determined in CBMCs. The results of Table 3 demonstrate a correlation between the level of resistance to 3TC and cross-resistance to the novel compounds tested. Isolate 3887 displayed moderate resistance to 3TC and a slight increase in IC_{50} for (+) dOTFC while isolate 4205 was highly resistant to 3TC and showed an increase in IC_{50} of >10-fold for each of the novel compounds.

DISCUSSION

Our results indicate that the M184I and M184V mutations in RT, that confer resistance to 3TC, may be selected with (+) dOTC and (-) dOTFC. This finding is not surprising since these compounds are structurally similar to 3TC. The pattern of 3TC resistance obtained correlates with the degree of cross-resistance to the novel compounds; M184I confers low-level resistance to both 3TC and the novel compounds, while M184V confer higher-level resistance in each case. In both patients undergoing 3TC monotherapy and in tissue culture selections, the M184I mutation appears first and is then replaced by the M184V mutation alongside an increase in IC_{50} value for 3TC[1]. It will be interesting to determine whether similar patterns are observed with the novel compounds.

While these results were obtained using the MT-4 cell line, previous studies performed in CBMCs used the HXB2D clone[9]. The level of resistance to the compounds observed is less in CBMC than in MT-4 cells. These differences may be due to differences in the rates of phosphorylation of the drugs and /or minor differences in the ability of the virus to replicate in different cell types[10]. The levels of resistance observed with clinical isolates were intermediate; cross -resistance was observed between 3TC-resistant isolates and the novel compounds, but not to as great an extent as observed with MT-4 cells. Thus, the M184I and M184V mutations confer resistance to the novel cytidine analogues tested although at lower much lower levels than associated with 3TC. Nonetheless this finding may be important when considering whether these compounds should be used in clinical trials.

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