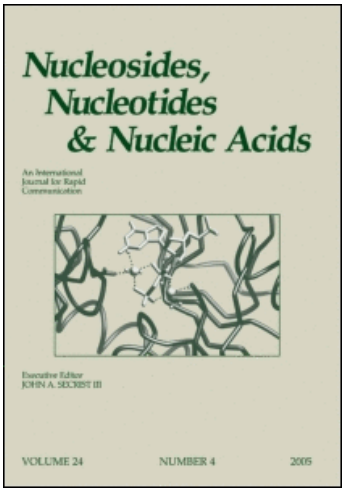

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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To cite this Article Richard, Nathalie , Belmonte, Antonietta R. , Salomon, Horacio , Nagai, Kazushige , Mansour, Tarek and Wainberg, Mark A.(1997) 'Resistance Profile of a Novel 2', 3' - Dideoxynucleoside Analog with Activity Against HIV-1', Nucleosides, Nucleotides and Nucleic Acids, 16: 7, 1235 — 1240
To link to this Article: DOI: 10.1080/07328319708006165
URL: http://dx.doi.org/10.1080/07328319708006165

NUCLEOSIDES & NUCLEOTIDES, 16(7-9), 1235-1240 (1997)

RESISTANCE PROFILE OF A NOVEL 2', 3' - DIDEOXYNUCLEOSIDE ANALOG WITH ACTIVITY AGAINST HIV-1.

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ABSTRACT. We have selected for resistance against a novel compound, (+) dOTFC, using HIV-1 tissue culture replication assays. A novel D67G (asp gly) mutation in HIV-1 reverse transcriptase has been identified that confers about 5-fold resistance to this drug but not to other nucleoside and non-nucleoside inhibitors of HIV-1 reverse transcriptase.

INTRODUCTION

Previous research has shown that a series of four novel heterosubstituted 2', 3'-dideoxynucleoside analogs possess activity against replication of the human immunodeficiency virus type 1 (HIV-1).[1,2] These structures are the (+) and (-) enantiomers of 2'-deoxy-3'-oxa-4'-thiocytidine (dOTC) and its fluorocytidine derivative, 2'-deoxy-3'-oxa-4'-thio-5-fluorocytidine (dOTFC). It was of interest to determine whether or not it might be possible to select for resistance against these compounds using tissue culture protocols. In addition, we wished to assess whether resistant viruses might display extensive cross resistance against other nucleoside and non-nucleoside inhibitors of HIV reverse transcriptase (RT) activity. These studies were performed as part of important pre-clinical investigations, to determine whether these novel nucleoside analogs should be considered for clinical trials.

MATERIALS AND METHODS

We employed MT-4 cells to grow both wild-type and resistant variants of HIV-1 as previously described.[3,4] In these protocols, the concentrations of drugs are gradually increased over approximately 12 passages in order to permit for the selection of resistant viruses. We extensively utilized the HxB2D clone of HIV in order to facilitate sequencing analysis conducted on resistant isolates. Determinations of IC_{50} values were carried out using both MT4 cells and peripheral blood mononuclear cells (PBMCs), obtained from healthy human donors, as previously described.[3,4]

For purposes of cloning, sequencing and site directed mutagenesis, total cellular DNA was harvested from MT-4 cells that had been infected with resistant variants of HIV derived from our tissue culture selection protocols. Cloning and sequencing studies to identify potentially novel mutations in the RT gene of HIV-1 were performed as previously described.[5] In addition, we introduced novel mutations that we identified into the HxB2D clone of HIV-1 by site-directed mutagenesis.[5] Subsequently, we employed such viruses in studies to directly determine whether any of the novel mutations identified displayed resistance in standard tissue culture protocols.

RESULTS

We initially sought to determine to what extent each of the four novel compounds would display anti-viral effects and whether or not they could antagonize HIV-1 variants previously shown to display resistance to other drugs. In this context, we employed each of wild-type HxB2D recombinant virus, as well as each of HxB2D-K65R and HxB2D-M184V, previously shown to be resistant against each of ddC and 3TC.[4,6,7] The results of Table 1 show that the K65R substitution displayed resistance against each one of these four novel heterosubstituted compounds. In contrast, the M184V mutation, that confers high-level resistance to 3TC,[6,7] conferred resistance to only two of these novel structures, (+) dOTC and (+) dOTFC.

Next, we sought to determine whether we could select, over time, for resistance against these compounds in MT-4 cells. The results of Table 2 demonstrate that resistance of 5-10 fold could be generated against three of the four drugs studied using previously described protocols.[3,4] In each case, viruses selected for resistance using MT-4 cells were assessed for IC_{50}values in PBMCs. Studies on the fourth compound revealed an inability of HIV to replicate in increasing drug concentrations.

TABLE 1. IC_{50} Values (M) of HIV-1 Isolates for Various Drugs in PBMC

Drug	HxB2D	HxB2D-K65R	HxB2D-M184V
AZT	0.002	0.001	0.001
3TC	0.08	0.125	42.5
(-)dOTC	1.0	2.5	0.53
(+) dOTC	0.1	3.5	3.0
(-) dOTFC	3.0	6.5	2.0
(+) dOTFC	3.0	11.5	9.0

TABLE 2. IC_{50} Values (M) Following Tissue Culture Selection for Resistance to Novel Compounds in PBMC

Compound	HxB2D	Drug-selected
(+) dOTC	0.3	1.6
(-) dOTFC	1.5	15
(+) dOTFC	1.0	12.5

Because previous studies had indicated that one of these novel structures, i.e. (+) dOTFC had a better selectivity index than the others, and was therefore the most likely to be developed for future clinical trials, we performed sequencing analysis on the RT genes of variants that displayed resistance against this drug. A total of five sequencing analyses were performed, and revealed a common D67G (asp -> gly) substitution in each case. We therefore generated, by site-directed mutagenesis, a recombinant virus containing this mutation. Because this virus was derived from the recombinant HxB2D wild-type clone of HIV-1, it was designated HxB2D-D67G. The results of Figure 1 demonstrate that this virus displayed resistance

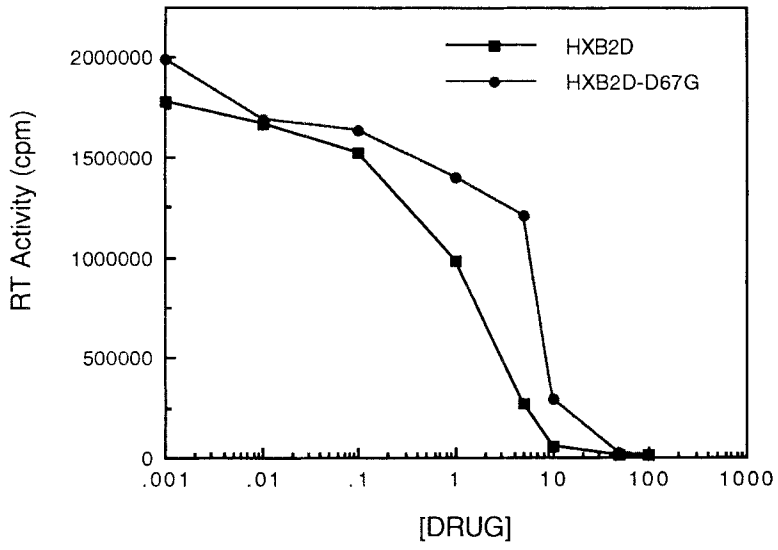

FIG. 1 Sensitivity curves of HxB2D and HxB2D-D67G to (+) dOTFC

against (+) dOTFC over a wide range of concentrations. In fact, the IC_{50} value of HxB2D in regard to (+) dOTFC is 1.4 μM while that of HxB2D-D67G is 6.5 μM.

Next, we asked to what extent this novel substitution, i.e. D67G, might confer cross-resistance against a variety of other nucleoside and non-nucleoside inhibitors of HIV-1 RT. In this regard, we studied each of AZT, 3TC, ddC, ddI, and d4T, all of which are well-described nucleoside analog antagonists of viral replication capacity. In addition, each of two non-nucleoside RT inhibitors (NNRTI) were studied, Delavirdine and Nevirapine. The results of Table 3 show that the IC_{50} values obtained for each of HxB2D and HxB2D-D67G did not differ significantly in regard to each of these drugs. Therefore, the D67G mutation, while conferring a modest level of drug resistance against (+) dOTFC, did not confer significant cross-resistance against the other RT inhibitors studied.

DISCUSSION

These findings suggest that (+) dOTFC should be considered for clinical development, as it possesses significant anti-HIV activity, yet displays little or no cross-resistance in regard to other nucleoside and NNRTI compounds studied. In particular, it is significant that no cross-resistance apparently exists

TABLE 3. IC_{50} Values (M) of HIV-1 Isolates for Different Drugs in PBMC

Drug	HxB2D	HxB2D-D67G
AZT	0.0048	0.0027
3TC	0.13	0.15
ddC	0.041	0.24
ddI	2.8	5.0
d4T	0.42	0.35
Delavirdine	0.023	0.041
Nevirapine	0.019	0.003

between (+) dOTFC and 3TC, a structurally different heterosubstituted nucleoside. We found that the M184V substitution in RT conferred resistance to only two of the four novel compounds studied, although the K65R substitution, previously shown to confer resistance against ddC,[4,8] was clearly able to display resistance against each of these novel drugs. These findings are consistent with previous observations that revealed a moderate degree of cross-resistance between each of ddC and 3TC in regard to both the M184V and K65R mutations.[4,8]

It is surprising that the novel D67G mutation, here identified, did not display resistance to 3TC, given the above-mentioned results. It is also puzzling that D67G did not confer resistance against ddC, the parent compound from which all novel heterosubstituted dideoxycytidine analogs are derived. Nor did the D67G substitution result in resistance to AZT, although an independent mutation at this position, i.e. D67N (asp asn), confers low-level resistance, i.e. 1-2 fold, to the latter compound.[9] In this context, it may be significant that our tissue culture findings were obtained using PBMCs. Further analysis will investigate whether cross-resistance might be demonstrated using a variety of T cell lines, e.g. Jurkat, MT4, MT2.

Finally, we will now try to express and purify recombinant reverse transcriptase proteins that contain the novel D67G substitution. This will be followed by research to assess whether such proteins are resistant against the triphosphate derivatives of the series of oxa-thiocytidine nucleoside analogs

described in this and previous manuscripts, as previously demonstrated for other RT inhibitors.[10,11]

ACKNOWLEDGMENTS

Research in our laboratories has been supported by grants from Health and Welfare Canada and by the Medical Research Council of Canada. Mark A. Wainberg is a national AIDS scientist of Health Canada. Nathalie Richard was supported by a pre-doctoral fellowship provided by Health Canada.

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