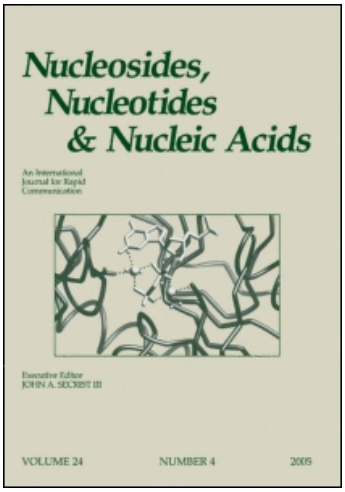

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In Vitro Study of Resistance-Associated Genotypic Mutations to Nucleoside Analogs

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ABSTRACT

In spite of a rather long period of investigations, the problem of HIV drug resistance remains unsolved, and more that, at present HIV-1 mutants resistant to all known nucleoside inhibitors being used in clinical therapy against the human immunodeficiency syndrome are discovered. In this study we selected HIV-1 mutants resistant to the nucleoside inhibitors of HIV reverse transcriptase (NRTI): 3′-azido-2′,3′-dideoxythymidine (AZT), 5′-phosphit 3′-azido-2′,3′-dideoxythymidine (ph-AZT), dideoxyinosine (ddI) and didehydrodeoxythymidine (d4T). Selection of resistant mutants was carried out by gradually increasing of drug concentration in the culture medium during propagation of the HIV-1_{EVK} on fresh MT-4 cells. Phenotypic resistance was defined as an increase in ID_{50} of 160-fold for AZT, 8 for ph-AZT, 10 for ddI, 7 for d4T. In comparison studies it was determined that the viral resistance to these drugs was appeared variously in a similar conditions and duration of selection. The nucleotide sequences of the RT region of the HIV-1 variants were compared with the HIV-1_{EVK} from "0" passage. For some of selected HIV-1 mutants NRTI resistance mutations were detected. Selected AZT resistant variants contained amino acid substitutions in

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positions D67A and K70R. Our studies was not revealed substitution at position 75 for ph-AZT resistant variants, whereas substitution at position L214F have been observed in both experiments using AZT and ph-AZT. Selected d4T resistant mutants contained amino acid substitutions in positions N54D and P52R. Selected ddI resistant mutants contained only one amino acid substitution in position P143S. Collection of drug-resistant mutants should prove to be a convenient tool for rapid investigations a new antiretroviral agents on cross drug-resistance.

Key Words: HIV-1 mutants: Drug resistance; Nucleoside reverse transcriptase inhibitors (NRTI).

The ability of human immunodeficiency virus type 1 to develop drug-resistance against a variety of antiretroviral agents is one of the general problems, limiting the effectiveness of most anti-HIV-1 therapies, so the emergence of drug-resistant variants of HIV continues to be of prime interest in the fields of HIV disease pathogenesis and antiretroviral chemotherapy.

The aim of this work was the investigation of drug resistance emergence and the creation of a collection of HIV-1 mutants resistant to the nucleoside inhibitors of HIV reverse transcriptase: 3′-azido-2′,3′-dideoxythymidine (AZT), 5′-phosphit 3′-azido-2′,3′-dideoxythymidine (ph-AZT), dideoxyinosine (ddI) and didehydrodeoxythymidine (d4T).

Selection of resistant mutants was carried out by gradually increasing the drug concentration in the culture medium, concerning with 1/3 ID_{50}, during propagation of the original strain HIV-1_{EVK}[1] on fresh highly HIV-sensitive MT-4 cells for 8–17 passages. Virus production in experimental and control samples was estimated by ELISA detection of virus-specific p24 protein, also the viable cells were detected by the tripan blue exclusion method.

Selected HIV-1 variants were characterized by decreased susceptibility to AZT 160-fold, to ph-AZT 8-fold, to ddI 10-fold, to d4T 7-fold. Data obtained are presented in Table 1.

We performed a comparative study of the rate of resistance development to AZT and ph-AZT in similar conditions and duration of selection. Experimental data obtained are presented in Table 2.

Table 1. The resistance levels for drug-resistant HIV-1 mutants.

		ID_{50}, μM		ID_{90}, μM		IS		
Drug	CD_{50}, μM	Wild virus	Selected virus	Wild virus	Selected virus	Wild virus	Selected virus	Resistance level
AZT	149,70	0,056	9,0	0,187	> 37,5	2673,2	16,63	160,8
ph-AZT	1180,48	0,236	1,84	1,495	> 262,3	5002	641,57	7,8
ddI	> 1693,27	12,69	131,23	43,61	> 380,98	> 133,4	> 12,9	10,3
d4T	521,86	0,312	2,23	3,35	> 4,46	1672,6	234	7,2

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Table 2. In vitro selection of drug-resistant HIV-1 mutants in MT-4 cells.

	Selection with AZT			Selection with ph-AZT		
Passage	Concentration (μM)	Run of passage (day)	p24 accumulated (ng/ml)	Concentration (μM)	Run of passage (day)	p24 accumulated (ng/ml)
1	0.00374	4	471.90	0.0131	4	183.58
3	0.00749	4	889.17	0.0262	4	75.95
7	0.03	4	1021.85	0.131	4	1961.64
9	0.0749	4	1156.48	0.262	4	170.67
11	0.15	4	606.25	1.049	4	1927.87
13	0.374	3	335.10	4.2	8	631.75
15	0.374	7	716.11	8.4	7	268.99
16	1.5	8	393.66	16.8	4	204.11
17	1.5	4	1063.50	–	–	–

From the results of this study it seems that the viral resistance to the drugs differs: upon passage 16 the AZT-resistant mutant was more highly resistant to the drug (160-fold), than the ph-AZT-resistant mutant (8-fold), selected over the same period. Experimental data of the inhibition of selected mutants by AZT and ph-AZT on the mutants are presented in Table 3. The AZT-resistant mutant showed a cross-resistance to ph-AZT (356-fold), whereas the ph-AZT-resistant mutant was more sensitive to AZT, than to ph-AZT.

From resistant HIV-1 variants, the part of the viral genome which encodes the RT was analyzed. DNA sequence analysis of the *pol* gene revealed that the AZT-resistant virus strain carries mutations at positions D67V (16,7%-clones carrying a substitution/clones screened), D67A (16,7%), K70R (55,6%) and L214F (38,9%). In turn, the ph-AZT-resistant virus strain has an amino acid substitution L214F (13,6%). It should be noted, however, that this mutation is somewhat less frequent in the material being studied. Comparison of the RT sequences of a resistant virus with those from a sensitive virus revealed mutations: N54D (61%), P52R (39%) for d4T and P143S (60%) for ddI.

Table 3. Sensitivity of drug-resistant HIV-1 mutants to antiviral drugs AZT and ph-AZT.

	ID_{50}, μM	
Virus	ph-AZT	AZT
$HIV\text{-}1_{EVK}$	0.236	0.0561
AZT-resistant mutant	84.00	9.00
Drug efficacy reduction, fold	356	160
ph-AZT-resistant mutant	1.84	0.15
Drug efficacy reduction, fold	8	2.6

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Overall, data provided herein indicate that the mutants resistant to ph-AZT, ddI, d4T are much more difficult to make than those resistant to AZT. Machado et al.[2] reported only one mutation in codon 67 for ph-AZT-resistant HIV-1 mutant. Our data that there is a mutation occurring in codon 214 of the *pol* gene of both AZT- and ph-AZT-resistant mutants, and the data that the AZT-resistant mutant possesses cross-resistance to ph-AZT suggests that this mutation might probably contribute to the development of resistance to ph-AZT. The mutation at position 214 of the AZT-resistant mutant had earlier been described only when the AZT/3TC combination was used.[3] Nevertheless, a rather high percentage of the clones revealed as carrying this mutation, indicates that these mutants are likely to occur even in the presence of either drug alone. Having the virus carrying this mutation alone cloned will help elucidate its role in the development of resistance to AZT and ph-AZT.

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