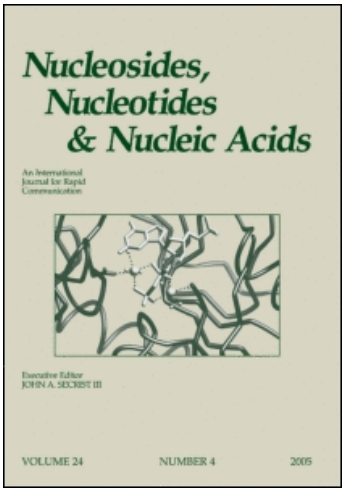

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ANTIVIRAL ACTIVITY AND RESISTANCE PROFILE OF PHOSPHAZID - A NOVEL PRODRUG OF AZT

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ABSTRACT: Both AZT and its novel 5'-hydrogen phosphonate derivative, Phosphazid, possess similar in vitro activity and resistance profiles. Experiments involving AZT-resistant virus isolates revealed a strong correlation between resistance to AZT and cross-resistance to Phosphazid. In vitro selection for resistance to Phosphazid yielded viruses that were about 15-fold less sensitive than wild-type virus to this drug. Sequencing of the reverse transcriptase region of seven Phosphazid-selected viruses revealed a single codon mutation, D67N, that is associated with resistance to AZT.

INTRODUCTION

3'-Azido-3'-deoxythymidine (AZT) is used extensively in the treatment of HIV infected patients but is prone to problems of drug resistance. The therapeutic usefulness of nucleoside analogs lies in their ability to effectively antagonize the viral encoded reverse transcriptase (RT) enzyme[1]. RT is essential for viral replication and is largely responsible for the vast genetic heterogeneity observed in HIV-1 populations. Obstruction of reverse transcription prevents the formation of a complete double stranded DNA pre-integration complex. In the case of HIV, failure to integrate into host chromosomal DNA equates to an inability to express crucial viral proteins necessary for replication.

Modifications to the phosphate moiety of AZT monophosphate (AZT-MP) have yielded a repertoire of novel nucleoside analogs that have been shown to possess potent

anti-retroviral activity. Phosphazid , a 5'-hydrogen phosphonate derivative of AZT, is one such compound and is currently undergoing phase I clinical trial testing in Russia.

For any new compound exhibiting anti-HIV activity, its corresponding in vitro resistance profile and selectivity index value can be useful indicators of future in vivo efficacy. To this end, we have extended earlier work[6,7] by investigating the selectivity index of Phosphazid in both the MT-4 cell line and primary cells. In addition, we have selected for resistance to Phosphazid in an in vitro system and have compared this with resistance development to AZT and 3TC. We have also sequenced the RT regions of seven Phosphazid-selected viruses for biologically-relevant mutations.

METHODS and MATERIALS

Virus and cells: Stocks of HXB2 or HIV-IIIB viruses were used to infect MT-4 and cord blood mononuclear cells as previously described.

In vitro selection of drug resistant variants[2-4]

Cells were infected with 200 TCID50 units of virus in the presence of low drug concentrations i.e. about one-fifth of the compound's IC50 value. Following incubation, cell-free supernatants, harboring progeny virus, were used to start another round of infection at a higher drug concentration. After many such passages, chromosomal DNA was extracted for the purpose of cloning and sequencing the RT regions of drug-selected viruses.

Cloning and sequencing of RT

Chromosomal DNA, containing HIV-1 proviral DNA, was extracted and a 1,742 bp region containing the complete RT sequence was amplified using two primers, RTO1 and RTO2[2,3]. Cloning of PCR-generated RT fragments was performed using a commercially available kit (Invitrogen Topo Original Cloning Kit, Invitrogen, Carlsbad, CA). Positive recombinant clones were subsequently subjected to automated DNA sequencing of the RT region.

RESULTS

Drug activity

The in vitro activities of Phosphazid and AZT were measured by determining the dose needed for 50% inhibition of wild type virus replication (IC50 value) in cord blood mononuclear cells (Table 1). An approximate 10-fold difference was apparent between the

TABLE 1. Inhibitory activity of AZT and Phosphazid against two strains of HIV in cord blood mononuclear cells.

Drug	IC50 for HIV-IIIB (uM)	IC50 for HXB2 (uM)	CCID50 (uM)	Selectivity Index for HIV-IIIB	Selectivity Index for HXB2
Phosphazid	0.036	0.017	1982	55055	116588
AZT	0.002	0.007	60	30000	8571

Values represent averages of duplicate studies.

TABLE 2. Sensitivity to Phosphazid and AZT of clinical isolates in CBMCs

Virus	Phosphazid		AZT	
	IC50 (uM)	Fold-resistance	IC50 (uM)	Fold-resistance
Wild Type				
HXB2	0.05	1	0.004	1
Clinical Isolates				
1073	0.1	2	0.0065	1.6
1074	0.3	6	0.037	9.3
1075	3.5	70	0.18	45
1082	30.7	614	1.8	450

IC_{50} values represent averages of duplicate tests.

IC50 of Phosphazid compared with that of AZT. Calculations of the ratio for each drug of 50% cell culture inhibitory dose (CCID50)/IC50 value showed that Phosphazid had a higher selectivity index than AZT.

Virus isolates obtained from patients on long-term AZT therapy were studied to assess whether AZT-resistant virus can exhibit cross resistance to Phosphazid. These viruses were known to contain most of the mutations associated with resistance to AZT. Table 2 shows the values obtained for each of these isolates in cord blood mononuclear cells (CBMCs). Resistance was observed in each case and to about the same extent, indicating that there is a strong association between AZT resistance and cross-resistance to Phosphazid.

TABLE 3. Summary of results of in vitro selection of drug-resistant HIV-1 variants in MT-4 cells.

Infection Cycle	Phosphazid Selection		AZT Selection		3TC Selection	
	Phosphazid Concentration (uM)	Duration (Days)	AZT Concentration (uM)	Duration (Days)	3TC Concentration (uM)	Duration (Days)
1	0	3	0	3	0	3
2	1	3	0.014	3	1.4	6
3	4	4	0.028	3	2.8	4
4	10	7	0.14	4	5.6	3
5	15	14	0.28	5	7	3
6	40	7	1.4	8	14	3
7	80	10	1.4	8	70	3
8	160	10	1.4	8	140	3
9	160	8	2	8	280	3
10	160	6	2	8	280	3
11	160	6			400	3
12	160	6				

Selection for resistance

We also selected for Phosphazid-resistant virus using the MT-4 cell line. Selections with AZT and 3TC were carried out concomitantly as positive controls. Initial drug concentrations were 1.4 μM, 1 μM and 0.01 μM for 3TC, Phosphazid and AZT, respectively (Table 3).

The sensitivity profile of the 3TC-selected virus showed a high level of resistance (i.e. IC50 > 200 mM), consistent with previous observations (Table 4). In contrast, AZT and Phosphazid selected viral populations were 10-15 fold above those of parental IC50 values. Phosphazid-selected virus had an IC50 value of 5.3 μM.

Cloning and sequencing of drug-selected proviral DNA

When the RT region (1,742 bp) of seven Phosphazid-selected viruses were subjected to single strand DNA sequencing, a substitution of a G to A (i.e. aspartate to asparagine) at codon 67 was observed in five of seven cases.

DISCUSSION

In this study, we have investigated the in vitro drug efficacy and resistance profile of Phosphazid in comparison with AZT. IC50, CCID50 and SI values obtained from assays

TABLE 4. IC50 values of wild-type HXB2 and drug-selected viruses.

Virus	IC50 (uM) 3TC	Phosphazid	AZT
Wild Type HxB2	0.5	0.35	0.02
Selected Virus	>200	5.3	0.2
Fold-Resistance	500	15	10

Values are averages of two or more studies.

performed in CBMCs were generally consistent with those previously obtained with MT-4 cells (5, 6). Phosphazid had a significantly higher selectivity index than AZT.

IC_{50} values for AZT and Phosphazid were calculated for four clinical isolates, 1073, 1074, 1075 and 1082, to evaluate whether AZT-resistant viruses might be cross-resistant to Phosphazid. The IC50s obtained for both AZT and Phosphazid show that cross-resistance was present between these two drugs. Moreover, resistance is apparently manifest at similar levels for both drugs.

We next selected for resistance against each of Phosphazid, AZT and 3TC in MT-4 cells. The IC50 value for virus-selected with Phosphazid over 84 days in culture was only 15-fold higher than that of parental virus.

Differential rates of development of resistance among drugs may be due to minor variations in culture conditions and manipulations required for selection with each compound. Of course, in the case of 3TC, rapid selection of resistance is expected due to the presence of the M184V substitution previously identified with this compound. The selectivity index of Phosphazid in comparison with AZT may make it a more attractive drug than AZT in the clinic, in spite of the shared resistance profiles of the two molecules. The fact that extensive selection with Phosphazid yielded only a single D67N substitution, also associated with resistance to AZT, rather than other AZT-resistance associated mutations as well, may also be a positive indication in regard to the potential of Phosphazid to combat HIV disease.

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