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Antiretroviral activity and cytotoxicity of novel zidovudine (AZT) derivatives and the relation to their chemical structure

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Abstract

Zidovudine (AZT) was the first nucleoside analogue licensed for the treatment of HIV infection. Efforts have continuously been made to improve the therapeutic characteristics of this drug, most of them focussed on prodrugs design. Here we describe the anti-HIV-1 activity and cytotoxicity of six novel AZT derivatives namely 3′-azido-3′-deoxy-5′-*O*-oxalyl-*N*-valinethymidine, 3′-azido-3′-deoxy-5′-*O*-oxalyl-*N*-leucinethymidine, 3′-azido-3′-deoxy-5′-*O*-oxalyl-*N*-isoleucinethymidine, 3′-azido-3′-deoxy-5′-*O*-oxalyl-*N*-phenylalaninethymidine, 3′-azido-3′-deoxy-5′-*O*-oxalylthymidine acid, 3′-azido-3′-deoxy-5′-*O*-isonicotinoylthymidine and 5-chloro-6-hydroxy-5,6-dihydro-3′-azido-3′-deoxythymidine which were perfectly characterized. AZT-Val, AZT-Leu, AZT-iLeu, AZT-Phen, AZT-Ac and AZT-Iso have shown a similar or higher selectivity index than that of AZT itself, in one or both of the different cell cultures used (PBMC and MT2). However, AZT-ClOH showed no anti-HIV activity. These results suggest that using amino acids in the design of AZT derivatives improves AZT activity.

Keywords: HIV; Antiretroviral activity; Zidovudine analogues; Cytotoxicity

1. Introduction

Human immunodeficiency virus type 1 (HIV-1) belongs to the family of retroviruses and is the cause of acquired immunodeficiency syndrome (AIDS). The primary target in fighting HIV-1 is the reverse transcriptase (RT), enzyme that plays a key role during the replicative cycle of the virus. Before the virus can be integrated into the host cell genome, a copy of the viral RNA has to be formed (proviral DNA) and this is regulated by this specific HIV DNA polymerase. If the DNA copy is not formed, the viral RNA genome becomes susceptible to destruction by cellular enzymes [1,2]. Several drugs that target the RT have been developed and can be divided into three groups: nucleoside analogues (NRTI), nucleotide analogues (NtRTI), and non-nucleoside analogues (NNRTI) [3,4].

Zidovudine, 3′-azido-3′-deoxithymidine (AZT) belongs to the NRTI group. It was the first drug licensed for clinical use and it is still used in highly active antiretroviral therapy (HAART) regimens, preventing mother-to-child transmission and in post exposure prophylaxis (PEP). AZT is a synthetic pyrimidinic analogue that differs from thymidine in having an azido substituent in place of the hydroxyl group at the 3′-position of the deoxyribose ring. Some major problems associated with AZT chemotherapy include bone marrow toxicity and suppression, low therapeutic index due to inhibition of cellular polymerases, low localization in brain and a short half-life in blood which requires frequent AZT administration to maintain a therapeutic drug concentration. Although AZT effectively inhibits the replication of HIV-1, it does not inhibit virus production by chronically infected cells or transmission of the infection to uninfected cells by syncitium forma-

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tion [5]. Moreover, patients under long-term therapy fail to control the infection because of the emergence of resistant variants of the virus.

Resistance to AZT was first documented in 1989. Viral isolates from patients with advanced HIV disease treated with zidovudine for a period of 6 or more months displayed a 100-fold decrease in their susceptibility to the drug [6]. Nucleotide sequence analysis of the HIV RT coding domain from both sensitive and resistant virus has indicated that resistance to AZT is associated with several substitutions at different codons, including 41, 67, 70, 210, 215 and 219 [7]. Recent studies have demonstrated transmission of phenotypic and genotypic resistance to AZT between individuals [8].

Prodrugs are pharmacologically inactive derivatives of active agents, which undergo chemical or enzymatic biotransformation resulting in the release of the active drug after administration. Prodrugs are designed to increase exposure of anti-HIV compounds, as well as to increase bioavailability and therefore, to achieve a simplification of both pharmacokinetic and posology. In attempts to overcome the problems of rapid elimination of AZT and to increase its therapeutic efficacy, numerous AZT prodrugs have been described in the literature ([9–14] reviewed in [5,15]). In most cases, the mechanism of action of these AZT prodrugs is based on hydrolysis of the enzymatically labile 5′-*O*-bonds between the drug (AZT) and its spacer group [16]. Regarding efficient anti HIV-1 agents, we have recently developed more lipophilic AZT derivatives with antiviral and bactericidal activity [17–20].

The aim of this work was to evaluate the antiretroviral activity and cytotoxicity on different cell cultures of six novel derivatives of AZT (Fig. 1) which present interesting lipophilic and protein binding properties as well as an effective action against aetiological agents of opportunistic infections associated with AIDS-complex [20,21].

2. Materials and methods

2.1. Chemistry

The nucleoside 3′-azido-3′-deoxythymidine (zidovudine, AZT), generously supplied by Filaxis (Buenos Aires, Argentina), was used without purification. The studied compounds 3′-azido-3′-deoxy-5′-*O*-oxalyl-*N*-valinethymidine (AZT-Val), 3′-azido-3′-deoxy-5′-*O*-oxalyl-*N*-leucinethymidine (AZT-Leu), 3′-azido-3′-deoxy-5′-*O*-oxalyl-*N*-isoleucinethymidine (AZT-iLeu), 3′-azido-3′-deoxy-5′-*O*-oxalyl-*N*-phenylalaninethymidine (AZT-Phen), 3′-azido-3′-deoxy-5′-*O*-oxalylthymidine acid (AZT-Ac), 3′-azido-3′-deoxy-5′-*O*-isonicotinoylthymidine (AZT-Iso) and and 5-chloro-6-hydroxy-5,6-dihydro-3′-azido-3′-deoxythymidine (AZT-ClOH) were synthesized as previously reported ([17,19,22] Motura MI. Ph.D. thesis 1998, School of Chemistry, National University of Córdoba, Córdoba, Argentina). Chemical structures of these compounds are shown in Fig. 1.

2.2. Cells and HIV-1 strain

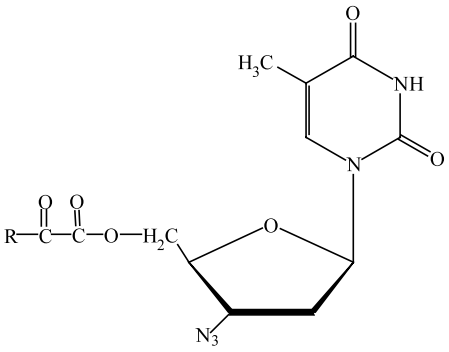
Two different cell cultures were used in this study. MT2 cells were cultured at 37 °C in RPMI-1640 medium (Sigma-Aldrich, USA) supplemented with 2 mM L-glutamine (Gibco BRL, USA), 100 U/ml penicillin (Gibco BRL), 100 mg/ml streptomycin (Gibco BRL) and 10% foetal calf serum (FCS, Gibco BRL). Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll–Hypaque (Amersham Pharmacia Biotech, Sweden) gradient centrifugation from peripheral blood of HIV-1 seronegative patients, stimulated with 0.1% phytohaemagglutinin (PHA) during 3 days and cultured in the same medium described as for MT2 plus 10 U/ml interleukin-2 (IL-2).

Stock of HTLV-IIIB strain of HIV-1 was derived from chronically infected H9 cells and was used in all assays described in this study.

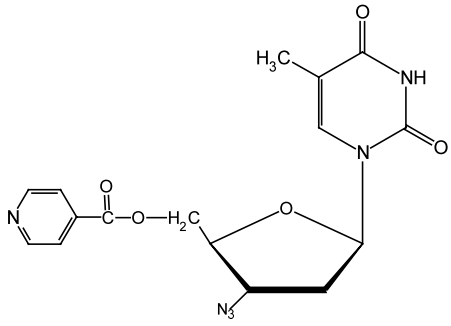
2.3. Antiviral activity and cytotoxicity assays

PBMCs were infected at 6.45×10^5 $TCID_{50}/10^6$ cells for 2 h at 37 °C. After infection, cells were washed and dispensed in a 96-well plate in the presence of various drugs concentrations. The experiments were performed in triplicate and wells treated with AZT were also monitored as controls of antiviral activity. Culture medium was changed at day 4 maintaining the original concentration of drug. On the seventh day, supernatant fluids were harvested and production of p24 antigen was subsequently evaluated using a commercial enzyme linked immunosorbent assay (ELISA) assay (ABBOT Laboratories, USA). Experiments on MT2 were performed at 1.3×10^5 $TCID_{50}/10^6$ cells. On the fourth day, the presence/absence of syncytia was observed and supernatant fluids were assayed for p24 antigen production. In lymphoblastoid cell cultures, the number of syncytia directly correlates with the production of p24 antigen, so a reduction in the number of syncytia present in the infected cell culture gives a rough idea of the inhibition of viral replication. Based on p24 assays, the dose that inhibited 50% of viral production (IC_{50}) was determined in both cases.

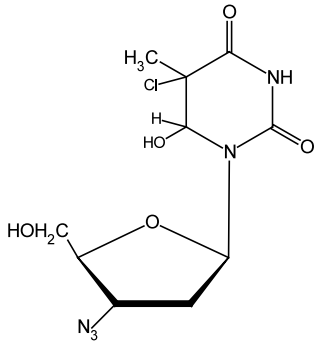
Cytotoxicity studies were performed in parallel on uninfected cells (PBMCs and MT2) in order to determine the concentration of drug that inhibited 50% of cell growth ($CCID_{50}$). Two different methodologies were used to evaluate the viability of the cells: trypan blue (TB) dye and subsequent counting of viable cells on Neubauer chamber, and sulforhodamine B (SRB) dye.



Compound	Drug R
AZT-Val	NH-CH(COOH)CH(CH$_3$)$_2$
AZT-Leu	NH-CH(COOH)CH$_2$-CH(CH$_3$)$_2$
AZT-iLeu	NH-CH(COOH)CH(CH$_3$)CH$_2$-CH$_3$
AZT-Phen	-NH-CH(COOH)CH$_2$C$_6$H$_5$
AZT-Ac	OH



AZT-Iso



AZT-ClOH

Fig. 1. Chemical structures of zidovudine (AZT) analogues.

Briefly, the latter methodology consists in fixing cells with TCA 80%, dyeing the fixed cells with SRB and measuring optical density at 492 nm [23]. Assays were performed in triplicate or quadruplicate when using TB method or SRB method, respectively.

Once both parameters ($CCID_{50}$ and IC_{50}) were obtained, the selectivity index (SI), which is defined as $CCID_{50}/IC_{50}$, was determined.

3. Results

Cytotoxicity and inhibition assays were performed in PBMC (primary cell culture) and in MT2 (cell line). Fig. 2 shows dose dependent cytotoxicity of each compound (except AZT-ClOH) corresponding to PBMC, for both methodologies used to evaluate viability of cells. As it can be seen, SRB dye is less sensitive than TB dye, but the former is by far less tedious to perform. Because of this, in spite of its reduced sensitivity (compared with TB dye), the SRB method can be used as a good approximation in cytotoxicity assays when the number of compounds to evaluate is high. Fig. 3 represents dose–response curves that show the inhibition of viral replication in PBMC caused by the compounds under study. In every case (except AZT-ClOH), a reduction in p24 antigen production was observed with increasing concentration of the drugs. Similar plots were obtained for both cytotoxicity and inhibition assays when using MT2 cells (graphics not shown; data shown in Table 1). In this case, the reduction in p24 antigen correlates with a reduction in syncytia formation

Based on the antiviral activity and the cytotoxicity values, we have calculated two Selectivity Indexes for each compound: SI^{TB} and SI^{SRB}, corresponding to both different methodologies used to evaluate cytotoxicity. Table 1 compares all parameters obtained for PBMC and MT2.

According to these results, and always comparing the SI determined for each compound and in each cell culture with those of AZT, we observe that AZT-iLeu shows a higher SI^{TB} and a similar SI^{SRB} in PBMC while in MT2 both parameters are lower. The same occurs for AZT-Leu. SIs^{TB} of AZT-Val and AZT-Ac are several folds higher than that of AZT, in PBMC as well as in MT2, while the SIs^{SRB} are similar. All parameters obtained for AZT-Iso are similar of those of AZT except the SI^{SRB} corresponding to MT2 cells, which is

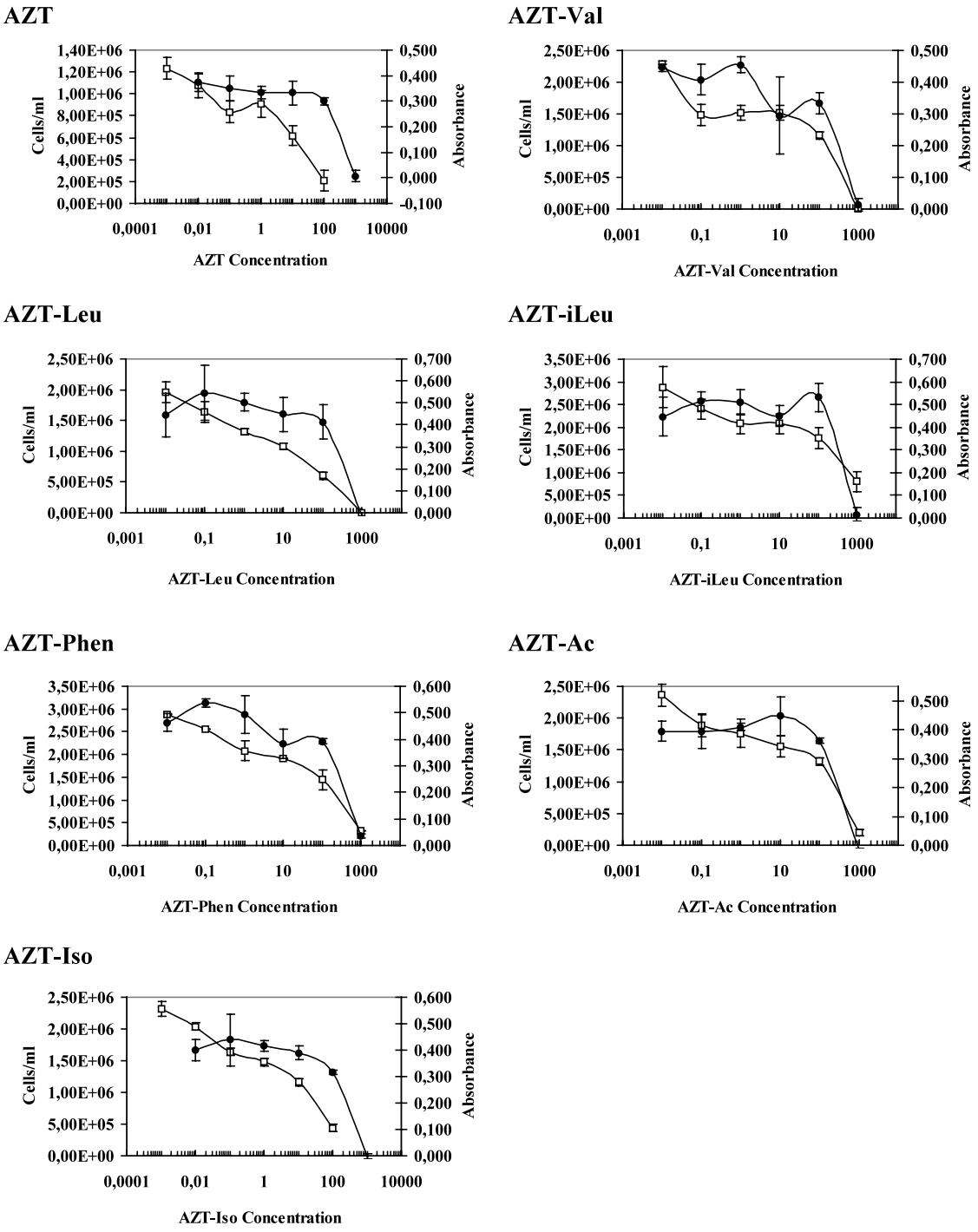


Fig. 2. Cytotoxicity testing of the novel compounds in PBMC. On seventh day, cytotoxicity was assayed using two different methodologies: tripan blue (TB) dye (white circles) and sulforhodamine B (SRB) dye (black squares). Values corresponding to the former methodology are represented on the left axes, while those corresponding to the latter are represented on the right axes. Assays were performed in triplicate or quadruplicate when using TB method or SRB method, respectively. Each point corresponds to the mean value ± S.D.

significantly lower. On the other hand, AZT-Phen showed lower parameters in each case evaluated. Assays with AZT-ClOH were discontinued after obtaining a $IC_{50} > 10$ μM in two different independent determinations, both on PBMC (see Fig. 3).

4. Discussion

As stated by Parang et al. (2000), more selective compounds can be designed by using the strategy of 5′-*O*-ester substituted AZT. Although the clinical applica-

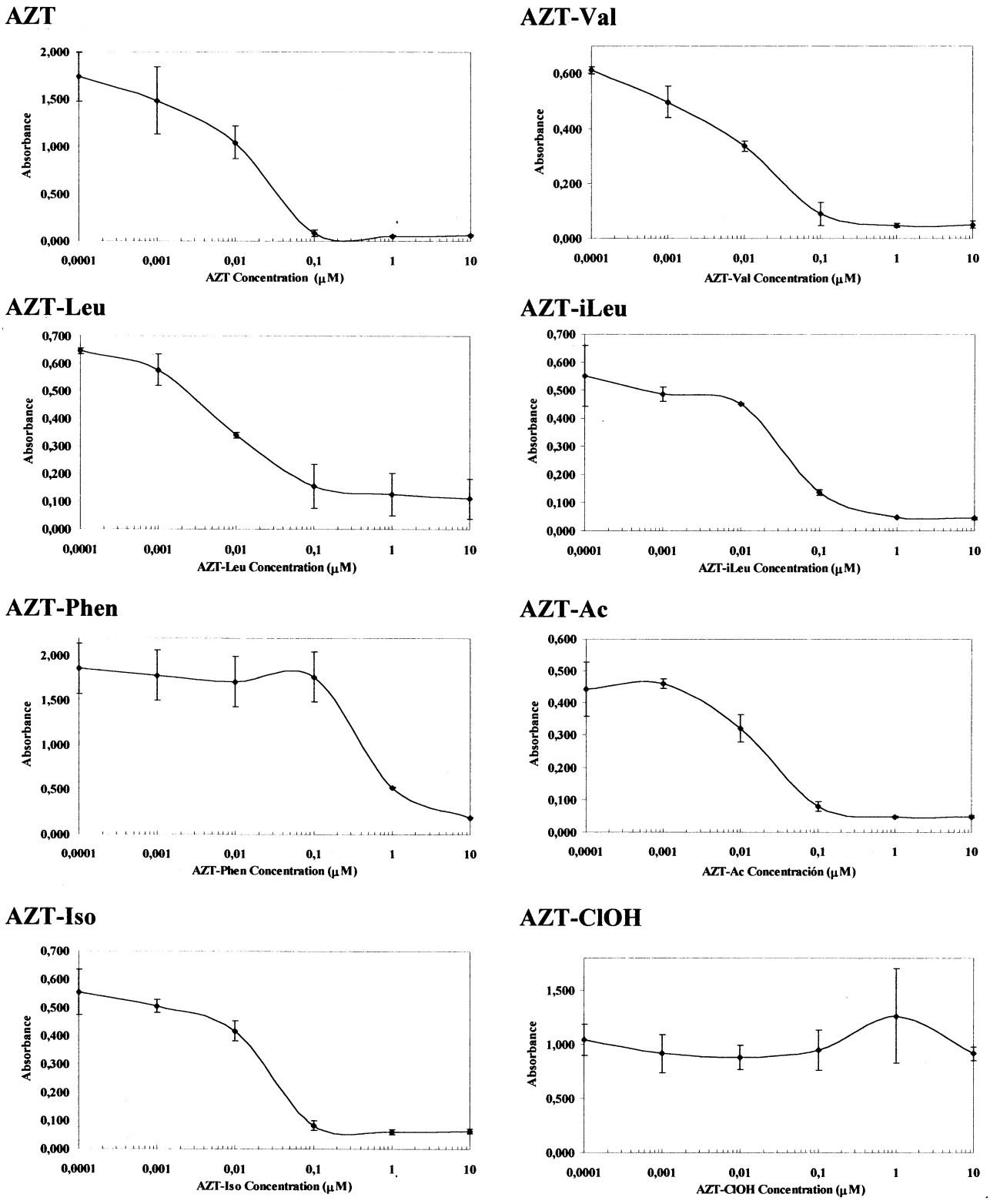


Fig. 3. Inhibitory effect of the AZT derivatives on HIV replication in PBMC. Assays were conducted in triplicate. On seventh day, supernatant fluids were harvested and production of p24 antigen was evaluated through a commercial ELISA assay. Curves show the decrease in p24 antigen production with increasing concentration of the drug. Each point corresponds to the mean value $\pm$S.D.

Table 1
Cytotoxicity, in vitro anti-HIV activity and selectivity indexes of the studied analogues of AZT, on both PBMC and MT2 cells

Drug	PBMC					MT2				
	$CCID_{50}$ (μM)[a]		IC_{50} (μM)[a]	SI[b]		$CCID_{50}$ (μM)[a]		IC_{50} (μM)[a]	SI[b]	
	TB	SRB		TB	SRB	TB	SRB		TB	SRB
AZT	10 ± 0.5	400 ± 86	0.02 ± 0.004	500 ± 100	20000 ± 5900	15 ± 0.57	350 ± 70	0.015 ± 0.001	1000 ± 77	24000 ± 4920
AZT-Val	100 ± 14	250 ± 50	0.015 ± 0.001	6700 ± 1000	17000 ± 3510	80 ± 5	500 ± 20	0.025 ± 0.001	3200 ± 240	20000 ± 1130
AZT-Leu	20 ± 1.4	300 ± 86	0.01 ± 0.001	2000 ± 240	30000 ± 9100	10 ± 0.7	500 ± 14	0.05 ± 0.002	200 ± 16	10000 ± 490
AZT-iLeu	200 ± 70	400 ± 86	0.035 ± 0.001	5700 ± 2000	12000 ± 2480	11 ± 2.8	450 ± 20	0.3 ± 0.07	37 ± 13	1500 ± 360
AZT-Phen	100 ± 5.6	300 ± 10	0.45 ± 0.07	250 ± 37	700 ± 110	150 ± 14	400 ± 70	0.35 ± 0.07	500 ± 95	1200 ± 300
AZT-Ac	150 ± 14	300 ± 10	0.025 ± 0.007	6000 ± 1770	12000 ± 3380	150 ± 10	400 ± 25	0.03 ± 0.007	5000 ± 1210	13000 ± 3220
AZT-Iso	10 ± 0.56	250 ± 50	0.025 ± 0.007	400 ± 110	10000 ± 3440	150 ± 9	400 ± 81	0.075 ± 0.007	2000 ± 220	5400 ± 1200
AZT-ClOH	ND	ND	> 10	ND	ND	ND	ND	ND	ND	ND

PBMC, peripheral blood mononuclear cells; $CCID_{50}$, 50% cell culture inhibitory dose; TB, tripan blue dye; SRB, sulforhodamine B dye; IC_{50}, 50% inhibitory concentration; SI, selectivity index ($CCID_{50}/IC_{50}$); ND, not determined.
[a] Results are shown as mean ± S.D.
[b] Error was calculated according to error propagation law.

tion of these approaches remains unknown, they hold the promise of becoming an important tool in treating HIV infection and its related consequences. In this study, we found that using certain aminoacids in a 5′-*O*-ester substitution strategy, it is possible to improve in vitro anti-HIV activity of AZT. It is especially interesting that this result is seen using two different methodologies to evaluate cytotoxicity. Although the parameters obtained when using one or the other methodology cannot be compared with each other, their correlation was significant.

We have specifically focussed the current discussion on the derivatives obtained from aminoacid association because of the very low activity showed by AZT-ClOH. We consider that this compound has to be converted back to a thymidine derivative with a double bound in C5–C6 of the base ring to release the activity. This transformation involves dissimilar mechanisms in relation to those required for other compounds which only need the hydrolysis of the group in the 5′-*O* position. Hence, the difference in the activity showed by AZT-ClOH could be explained by its low capability to recover the double bond in the pyrimidinic ring previously mentioned rather than the characteristic of the substituent.

AZT-Phen showed a diminished capacity to inhibit HIV replication which might be associated with the high acidity of its aminoacid or to its major volume. However, neither the acidity level nor the volume, account for the activity variation showed by the rest of the compounds. This could be due to different transport mechanisms that might be implicated in each case depending on either the aminoacid involved (for special transport) or the lipophilicity of the substituent.

Assays on PBMCs showed that the most active compounds were AZT-Val, AZT-Leu, AZT-Ac and AZT-iLeu ($IS^{TB} = 6700$, 2000, 6000 and 5700; $IS^{SRB} =$ 17 000, 30 000, 12 000 and 12 000, respectively) while AZT-Iso ($IS^{TB} = 400$; $IS^{SRB} = 10\,000$) were slightly less active. Experiments on MT2 cells demonstrated that the studied compounds have an interesting activity in the following order: AZT-Ac > AZT-Val > AZT-Iso > AZT > AZT-Phen > AZT-Leu > AZT-iLeu or AZT > AZT-Val > AZT-Ac > AZT-Leu > AZT-Iso > AZT-iLeu > AZT-Phen, taking into account SI^{TB} or SI^{SRB}, respectively. According to the results previously expressed, AZT-Val and AZT-Ac demonstrated a much improved capacity to inhibit HIV replication, both in PBMC and MT2 cells.

Even though the results shown in this study are encouraging, further assays still need to be conducted. Biodistribution, permeability and metabolization assays will help us understand the mechanism of action of these novel compounds. The resistance profiles that these compounds select, as well as the activity against resistant clinical isolates, are also important parameters that need to be determined.

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