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Short communication

Inhibitors of CTP biosynthesis potentiate the anti-human immunodeficiency virus type 1 activity of 3TC in activated peripheral blood mononuclear cells☆

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Abstract

Unlike hydroxyurea, the CTP synthetase inhibitor acivicin and, to a lesser extent, two other inhibitors of CTP synthesis, increased the phosphorylation and anti-HIV-1 activity of 3TC in PHA-P-activated PBMC. These data suggest that to improve the antiretroviral activity of 3TC, it may be worth focusing on inhibition of CTP synthesis.

Keywords: 3TC; CTP synthetase; HIV; PBMC

Highly active antiretroviral therapy (HAART) has been shown to control human immunodeficiency virus (HIV) replication efficiently, resulting in a decrease in morbidity and mortality rates. However, the failure of HAART to eradicate this retrovirus completely in infected patients has highlighted the importance of developing new antiretroviral treatments. The anti-HIV activity of the nucleoside reverse transcriptase inhibitors (NRTIs) that are part of HAART depends on intracellular metabolism to generate triphosphate derivatives, which compete with the corresponding endogenous triphosphates (deoxynucleoside triphosphates; dNTPs). Intracellular concentrations of dideoxynucleoside triphosphates (ddNTPs) and dNTPs are therefore critical in determining the antiviral efficiency of the NRTIs. The de novo synthesis of dNTPs is regulated by ribonucleotide reductase (RNR). Hydroxyurea (HU), a well-known RNR inhibitor that decreases the amount of endogenous dNTP, inhibits viral replication in vitro in monocyte-derived macrophages and dendritic cells (Piccinini et al., 2002) and acts in synergism with NRTIs (Gao et al., 1995). In HIV-infected patients, the long-term use of HU is hampered by hematopoietic toxicity and exacerbation of the toxic effects of NRTIs (Farrell, 2000; Lafeuillade et al., 2002). However, HU and other metabolic inhibitors could be useful in certain situations, such as salvage regimens (Kulkosky et al., 2002), resource-poor settings (Stephenson, 2001), and structured treatment interruptions (Garcia et al., 2003).

This cytostatic or anti-metabolite strategy has been extended to other key enzymes in nucleotide synthesis, depending on the structure of the NRTI (Balzarini, 2000). In this study, we focused on lamivudine [β-L-(−)-2′,3′-dideoxy-3′-thiacytidine, (3TC)], which is used to treat HIV infection and chronic hepatitis B (Farrell, 2000). As 3TC is a deoxycytidine analog, its antiviral activity might be increased by metabolic inhibitors targeting dCTP biosynthesis. We combined lamivudine with inhibitors of enzymes operating on different steps in the dCTP biosynthesis pathway, namely brequinar, 6-azauridine and acivicin, which target dihydroorotate dehydrogenase, OMP decarboxylase and CTP synthetase, respectively (Levine et al., 1980; Lyons et al., 1990; McLean et al., 2001). In this study, we compared the effects of these inhibitors on the antiviral activity of 3TC, and the amount of 3TC-TP formed and the 3TC-TP:dCTP ratio in human peripheral blood mononuclear cells (PBMCs).

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Peripheral blood mononuclear cells were separated from a buffy-coat from a healthy HIV-, HCV-, HBV-seronegative blood donor by Ficoll-Hypaque density gradient centrifugation. They were activated by incubation with 1 μg/ml phytohemagglutinin-P (PHA-P; Difco Laboratories) for three days in RPMI 1640 medium (InVitroGen) supplemented with 10% heat-inactivated fetal calf serum (FCS, +56 °C for 45 min; BioWest), 2 mM L-glutamine (Life Technologies) and 1% antibiotic cocktail (penicillin, streptomycin, neomycin; InVitroGen). Various concentrations of the drugs tested were then added to the cell cultures. After 24 h, the PHA-P-activated PBMCs were infected with 75 $CCID_{50}$ (50% cell culture infectious dose) of the reference lymphotropic HIV-1-LAI strain. Seven days after infection, we quantified HIV replication by measuring RT activity in cell supernatants (RetroSys®, Innovagen), and assessed the cytotoxicity of the drugs used alone or in combination, in uninfected cell cultures, by means of the trypan blue exclusion assay. We analyzed 50% effective concentration (EC_{50}) values and combinatory indices (CI), using the software developed by Chou and Talalay (1984).

HIV-1-LAI replication in PHA-P-activated PBMC was inhibited by 3TC, with an EC_{50} of 65 nM (Fig. 1A). 3TC also inhibited viral replication when combined with 1 μM brequinar, 10 μM 6-azauridine and 10 μM acivicin (Fig. 1A). These were the highest concentrations that were not cytotoxic to PHA-P-activated PBMC. Under our experimental conditions, only acivicin had a significant direct effect on HIV when used alone, decreasing viral replication by 75% (acivicin alone: 7580 ± 262 pg/ml versus untreated control: 31,125 ± 920 pg/ml). For two-drug combinations, HIV replication with the metabolic inhibitor alone was taken as the 100% control. Brequinar and 6-azauridine increased the anti-HIV effects of 3TC slightly (EC_{50} values: 12 and 47 nM, respectively, for 3TC:brequinar and 3TC:6-azauridine combinations). The antiviral activity of 3TC was strongly potentiated in the presence of acivicin; EC_{50} value equal to 1.5 nM (versus 65 nM for 3TC alone; Fig. 1A). These effects of acivicin on 3TC were confirmed by calculating combinatory indices, as previously reported (Clayette et al., 1997). The 3TC:acivicin combination was clearly synergistic (CI < 1; Fig. 1B). We also assessed the effects of HU on the anti-HIV activity of 3TC and ddI in the same experimental model. No effect was found with 3TC (EC_{50} value equal to 60 nM for 3TC:HU versus 65 nM for 3TC alone; Fig. 1A), and synergistic effects were observed with ddI (CI < 1; Fig. 1B). Acivicin, which is not expected to interfere with dATP metabolism, had no effect on the anti-HIV activity of ddI (CI ≥ 1, data not shown).

We investigated the phosphorylation of 3TC to generate its triphosphate derivative, 3TC-TP, using radiolabeled 3TC (0.1 μM 2.5 μCi [5-^{3}H]3TC; Moravek Biochemicals), in the presence or absence of metabolic inhibitors used at the concentrations indicated above. Intracellular levels of dCTP and of mono-, di- and triphosphate derivatives of 3TC were determined 24 h after the start of treatment, corresponding to the time of HIV infection in the antiviral assay. Briefly, PHA-P-activated PBMCs were collected by centrifugation and lysed with 60% methanol. Cell extracts were then heated for 2 min at +95 °C and placed at −20 °C overnight. The extracts were centrifuged and one-fifth of the supernatant was withdrawn for dNTP determination. The rest of the supernatant was used for the determination of intracellular concentrations of 3TC and its metabolites, as described by Cammack et al. (1992) with some modifications. HPLC separations were performed on an ion-exchange

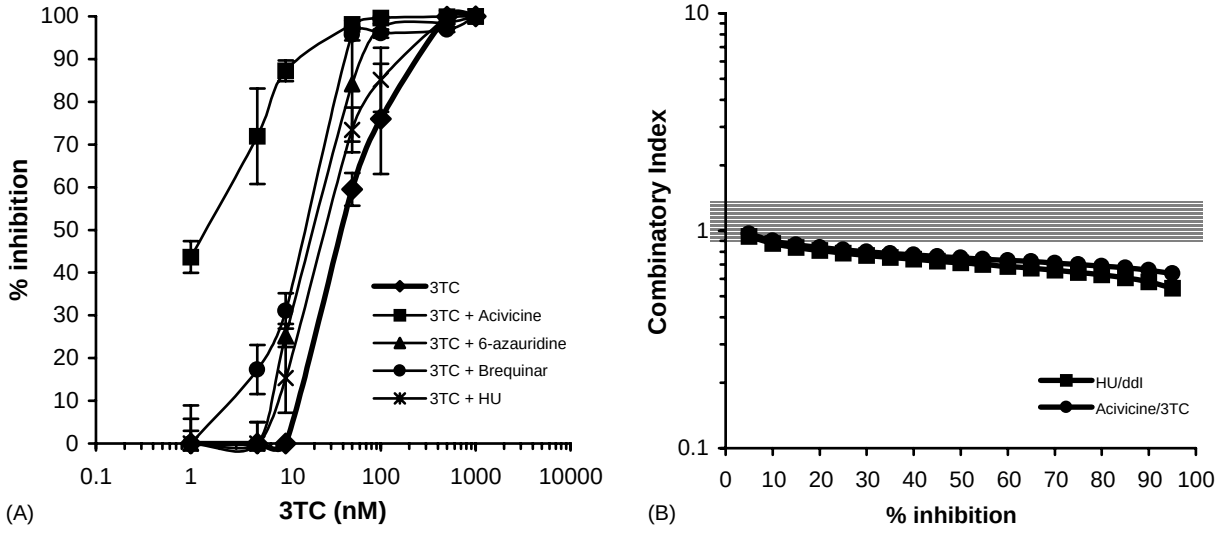

Fig. 1. (A) Effects of CTP biosynthesis inhibitors (acivicin and 6-azauridine at 10 μM, brequinar at 1 μM) and HU (100 μM) on the anti-HIV-1 activity of 3TC in PHA-P-activated PBMCs: dose–effect curves and (B) combinatory indices with respect to percent inhibition of HIV activity for 3TC combined with acivicin (CI < 1: synergistic effects; CI = 1: additive effects; CI > 1: antagonism). Drugs were added 24 h before infection and viral replication was measured seven days later. Experiments were performed in triplicate and are representative of two independent experiments performed with PBMCs isolated from two other blood donors.

Partisil 10-SAX column. The eluting buffers were 0.01 M potassium phosphate pH 3.5 (buffer A) and 0.5 M potassium phosphate pH 3.5 containing 0.8 M KCl. The elution program of 3TC and metabolites was 8 min of buffer A followed by a 20-min linear gradient to 100% buffer B, and a 10-min isocratic elution with 100% buffer B. The flow rate of the elution buffer was 1 ml/min. Radioactivity was monitored by running Quickszint flow 306 scintillator (Zinsser Analytic) through a radiomatic detector at a ratio of 2:1. The retention times of the labeled compounds were calibrated using unlabeled 3TC-MP, 3TC-DP, and 3TC-TP, generously provided by C. Guerreiro (Pasteur Institute, Paris). Metabolites of ddI were analyzed as described by Ahluwalia et al. (1987).

Cellular dNTP pools were quantified using elongation of ^{32}P 5′-end-labeled oligonucleotide primers annealed to complementary oligonucleotide templates, by the Klenow fragment of *Escherichia coli* DNA polymerase I (Roy et al., 1999). The elongated products were separated on a 10% polyacrylamide-urea gel, followed by autoradiography and densitometric analysis. We verified that 3TC-TP and ddATP did not interfere with the assay, for concentrations in cellular extracts of 3TC- and ddI-treated PBMC.

3TC was efficiently phosphorylated in PHA-P-activated PBMC (3TC-MP: 0.054 ± 0.014 pmol per million cells, 3TC-DP: 0.28 ± 0.03 pmol per million cells, and 3TC-TP: 0.33 ± 0.04 pmol per million cells), suggesting that, in this cell population, expression of the cellular kinases involved in the 3TC phosphorylation is either sufficient (e.g. 3-phosphoglycerate kinase (PGK); Krishnan et al., 2002) or increased (e.g. UMP-CMP kinase; Schinazi et al., 1994 and NDPK; Moore et al., 1999) by the mitogenic activation. Consistent with previous studies (Gao et al., 1994a), dCTP synthesis was not affected by 3TC treatment (Table 1). However, the dCTP pool was significantly smaller if 3TC treatment was combined with brequinar, 6-azauridine or acivicin (Table 1). This decrease in the amount of dCTP present was associated with an increase in 3TC-TP phosphorylation (not significant in the case of 6-azauridine) and, therefore, with an increase in the 3TC-TP:dCTP ratio and the anti-HIV effects of 3TC.

We tested HU as a control in our experimental model. Consistent with previously published data, HU increased the ddATP:dATP ratio in vitro (0.27 ± 0.03 versus 0.15 ± 0.05; $P < 0.05$) (Gao et al., 1995) and the anti-HIV activity of ddI (CI < 1) (Gao et al., 1994b). In contrast, HU did not affect 3TC-TP:dCTP ratio (Table 1), and did not potentiate the anti-HIV activity of 3TC (data not shown). In PHA-P-activated cells, two mechanisms have been proposed to account for the increase in anti-HIV-1 activity of NRTIs in the presence of HU. For ddI, this increase in activity results from a specific depletion of dATP. As suggested by Gao et al. (1995) and Hoggard et al. (2002), for pyrimidine nucleoside derivatives such as ddC and 3TC, HU may increase anti-HIV effects by increasing NRTI phosphorylation via activation of the pyrimidine salvage pathway. Gao et al. reported an increase in the amount of intracellular ddCTP associated with an increase in deoxycytidine kinase (dCK) activity in PHA-P-activated PBMC (Gao et al., 1995). Hoggard et al. (2002) reported a significant increase in dCTP levels in HIV-infected patients treated with 3TC and HU, although a significant decrease in 3TC-TP levels and 3TC-TP:dCTP ratio was observed in parallel. Consistent with these in vitro and in vivo data, we observed expansion of the dCTP pool, confirming involvement of the salvage pathway in biological effects of HU on the intracellular nucleotide pool. Nevertheless, in our experimental model, the activation of this pathway was not sufficient to increase the 3TC-TP:dCTP ratio and anti-HIV activity of 3TC. Unlike HU, acivicin, brequinar, and 6-azauridine, decreased dCTP levels in PHA-P-activated PBMC. This suggests that these inhibitors did not activate the pyrimidine salvage pathway efficiently or that they inhibited de novo dCTP synthesis more strongly than HU. The level of dephosphorylation or deamination of dCMP may also be higher in cells treated with pyrimidine inhibitors acting upstream from RNR. As 3TC phosphorylation is catalyzed, at least in part, by PGK (Krishnan et al., 2002), this process may also be less sensitive to the fluctuations induced by mitogenic activation of salvage pathway kinase production.

Overall, our data suggest that 3TC efficacy could be increased by combining the cytosine derivative with inhibitors of de novo pyrimidine biosynthesis, such as acivicin. Increases in the anti-HIV activity of 3TC in the presence of 3-deazauridine, another CTP synthetase inhibitor, have been reported (Gao et al., 2000). Similarly, Hoggard et al. (2002) also reported that patients in whom HAART was not successful had significantly lower 3TC-TP levels and 3TC-TP:dCTP

Table 1
Cellular pools of dCTP and 3TC-TP in uninfected, PHA-P-activated PBMCs incubated for 24 h with the indicated drugs

Treatment	3TC-TP (pmol per million viable cells)	dCTP (pmol per million viable cells)	3TC-TP/dCTP
None		2.95 ± 0.40	
3TC (0.1 μM)	0.33 ± 0.04	3.08 ± 0.19	0.107 ± 0.013
3TC/brequinar (1 μM)	0.47 ± 0.01**	1.80 ± 0.18*	0.261 ± 0.026*
3TC/6-azauridine (10 μM)	0.36 ± 0.03	1.76 ± 0.12*	0.204 ± 0.024*
3TC/acivicin (10 μM)	0.54 ± 0.05*	2.18 ± 0.19**	0.248 ± 0.029*
3TC/HU (100 μM)	0.44 ± 0.01**	4.35 ± 0.29*	0.101 ± 0.006

Data are means ± S.D. of three experiments performed in duplicate (* $P < 0.01$ or ** $P < 0.05$ versus the control value in Student's t test).

ratios than those observed in successfully treated patients. This highlights the importance of increasing the amount of 3TC-TP and the 3TC-TP:dCTP ratio by acivicin treatment in PBMC cultures. Acivicin is a glutamine antagonist that irreversibly inactivates several glutamine amidotransferases, including GMP synthetase and γ-glutamyl transpeptidase (Loh and Kufe, 1981; Lyons et al., 1990). Given the differential potency of acivicin for its various target enzymes, at the low concentration of this compound used in the present study CTP synthetase would preferentially be inhibited over, for example, GMP synthetase. Acivicin displays anti-tumor activity, but has also been shown to have undesirable gastrointestinal and neurological side-effects (for review, see Miles et al., 2002). These deleterious effects are likely to preclude, or to severely curtail, the in vivo applications of this drug. However, they seem to justify the development of new, safer CTP synthetase inhibitors, potentially as effective as acivicin at limiting HIV replication and increasing the antiviral effects of 3TC.

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