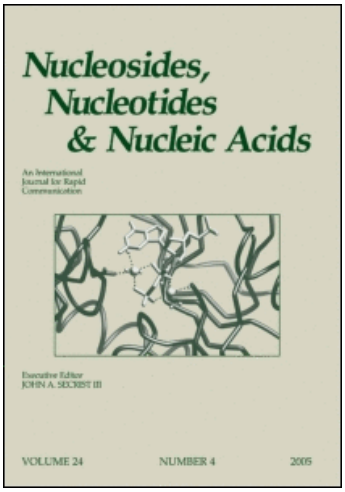

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POTENTIATION OF THE ANTI-HIV ACTIVITY OF ZALCITABINE AND LAMIVUDINE BY A CTP SYNTHASE INHIBITOR, 3-DEAZAURIDINE

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Dedicated to the memory of Dr. Gertrude B. Elion

ABSTRACT: Low levels of the CTP synthase inhibitor 3-deazauridine (3-DU) strongly potentiated the anti-HIV-1 activity of the 5'-triphosphates of the cytidine-based analogues [-]2'-deoxy-3'-thiacytidine (3TC; lamivudine) and 2',3'-dideoxycytidine (ddC). The potentiation was associated with a 3-DU-induced decrease in dCTP pool size; no changes were seen in cellular pool sizes of dATP, dGTP or dTTP.

The cytidine analogues lamivudine (3TC) and zalcitabine (ddC) are potent inhibitors of human immunodeficiency virus type-1 (HIV-1) reverse transcriptase[1,2] and hence of HIV-1 replication. In addition, 3TC shows significant activity in inhibiting replication of hepatitis B virus (HBV),[3] and is now in general use in the treatment of HBV infection.

In initial studies on potentiating the anti-HIV activity of dideoxynucleosides (ddNs) by depleting the pool size of the corresponding physiological dNTPs (dATP, dCTP, dTTP and dGTP), we noted that the ribonucleotide reductase inhibitor hydroxyurea (HU), while highly effective in enhancing the activity of purine-based ddNs such as 2'-β-fluoro-2',3'-dideoxyadenosine[4] and 2',3'-dideoxyinosine (ddI), was much less effective in the potentiation of pyrimidine-based nucleosides such as ddC and zidovudine (AZT).[5] On further investigation, we found that the marked potentiation of ddI by HU was due, not to an increase in the intracellular concentration of the active metabolite of ddI (ddATP) but rather to a specific depletion of the pool of the competing physiological dNTP, dATP. With AZT and ddC, on the other hand, the intracellular concentrations of the active metabolites

AZTTP and ddCTP increased after HU exposure, but an almost parallel increase in the pools of corresponding dNTPs (dTTP and dCTP) occurred simultaneously, largely negating any enhanced anti-HIV activity.[6] This effect of low concentrations of the ribonucleotide reductase inhibitor HU, i.e., of depleting dATP pools while increasing pools of the pyrimidine-based dNTPs, dTTP and dCTP, has been described by several previous workers and has been extensively explored and characterized by Bianchi and her colleagues[7,8] and more recently by Hendricks and Mathews.[9]

It became apparent, therefore, that successful potentiation of the anti-HIV activity of ddNs by this general method would require significant depletion of the pool of the appropriate corresponding competing dNTP, although without an obligatory requirement for an accompanying increase in the intracellular concentration of the pharmacologically active ddNTP; and that more selective agents would thus be needed to deplete pools of pyrimidine-based dNTPs. We have recently reported the application of this strategy to the thymidine-based anti-HIV agents, AZT and 2',3'-didehydro-2',3'-dideoxythymidine (D4T; stavudine), with dTTP pools being reduced through the use of thymidylate synthase inhibitors;[10] and, as reported below, have now extended these studies to the anti-HIV cytidine analogues ddC and 3TC, utilizing a prototypical CTP synthase inhibitor, 3-deazauridine (3-DU), in an attempt to deplete the pool of CTP and thus of the relevant competing dNTP, dCTP.

EXPERIMENTAL PROCEDURES

Materials. All chemicals used were of reagent grade. Phytohemagglutinin (PHA) was obtained from Sigma Chemical Co. (St. Louis, MO). ddC, 3TC and 3-DU were supplied by the late Dr. Karl Flora (Developmental Therapeutics Program, National Cancer Institute). Recombinant interleukin-2 was purchased from R&D Systems (Minneapolis, MN). Radioimmunoassay kits of p24 Gag protein were purchased from DuPont (Boston, MA). Sequenase enzyme (2.0 version) was obtained from United States Biochemical Corp. (Cleveland, OH). All synthetic oligonucleotides used as template primers were purchased from Genosys Biotechnologies (Woodlands, TX).

Cells and virus. Peripheral blood mononuclear (PBM) cells were isolated from heparinized venous blood of healthy donors and were incubated with PHA (10 μg/ml) for 48 hr in RPMI 1640 medium supplemented with 15% heat-inactivated fetal calf serum, 15 U/ml recombinant interleukin-2, 4 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin. An HIV-1 clinical strain ($ERS104_{pre}$) was isolated as previously described[11] from a patient with advanced HIV-1 infection before antiviral therapy.

Determination of anti-HIV-1 activity. PHA-stimulated PBM (PHA-PBM) cells were plated onto 24-well tisssue culture plates at a density of 1 x 10^6 cells/well. Drugs were added in 2 ml of supplemented RPMI 1640 medium. After incubation for 24 hr, cells were exposed to 2500 HIV-1 50% tissue culture infective doses/well. On day 7, the medium was harvested and the amount of p24 protein was determined by radioimmunoassay.

Deoxycytidine kinase assays. Intracellular levels of deoxycytidine kinase activity were determined as previously described.[12]

Determination of intracellular dNTP pools. Intracellular dNTP pools were determined by a DNA polymerase assay as previously described.[13,14]

RESULTS

Effect of 3-DU on inhibition by 3TC and ddC on replication of HIV-1 in PHA-PBM cells. Anti-HIV-1 activity was examined over the concentration range 0 - 0.10 μM for 3-TC and over the concentration range 0 - 0.04 μM for ddC. In the absence of 3-DU, inhibition of HIV-1 replication by these agents was 33 ± 6% for 0.1 μM 3TC and 30 ± 8% for 0.04 μM ddC. The addition of 0.5 μM 3-DU to the incubation mixture increased inhibition of HIV-1 replication to 51 ± 4% for 0.1 μM 3TC and to 59 ± 3% for 0.04 μM ddC. Complete (>99%) inhibition of replication was observed with both agents at these concentrations when the 3-DU level was increased to 1 μM (**FIG. 1**). In control studies, no anti-HIV activity was observed on incubation with 3-DU alone over the concentration range 0 - 1 μM.

Effect of 3-DU on dNTP pool size in PHA-PBM cells. Exposure of PHA-PBM cells to 3-DU (2 μM) reduced dCTP levels to 74 ± 3% of control levels at 24 hr and to 62 ± 3% of control levels at 48 hr, but had no detectable effect on pool sizes of dGTP, dTTP or dATP (**FIG. 2**).

Effect of 3-DU on host cell replication rate and on intracellular deoxycytidine kinase activity. In these studies, PHA-PBM cell numbers approximately doubled over 96 hr of incubation in the absence of 3-DU. Addition of increasing concentrations of 3-DU to the incubation medium (0.01 - 1 μM) resulted in slight but reproducible dose-related inhibition of the PHA-PBM cell replication rate (**TABLE 1**). More significant inhibition of growth rate (67.2% at 96 hr) was seen at the highest 3-DU concentration examined (10 μM) (**TABLE 1**). In control studies, i.e., in the absence of 3-DU, intracellular deoxycytidine kinase activity increased 2.4-fold in 20 hr (from 1.7 to 4.2 nmoles dCMP generated/hr/mg protein), an effect we have noted previously in PHA-PBM cells over extended incubation periods.[6] A slightly greater increase in enzyme activity (to 5.8 nmoles/hr/mg protein) was seen in the presence of 3-DU, 2.0 μM, and appeared to be dose-related (**TABLE 2**).

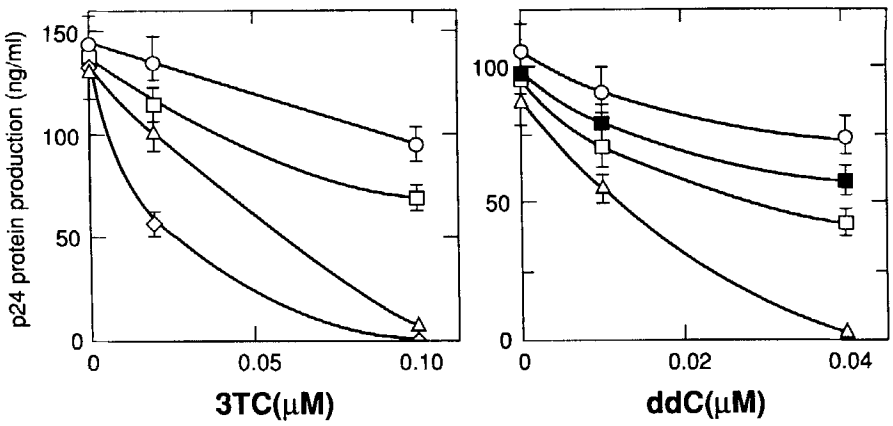

FIG. 1 (Left panel): Effect of 3-DU on inhibition by 3TC of the replication of an HIV-1 clinical strain ($ERS104_{pre}$) in PHA-PBM cells from normal donors. Assays were performed as described in **Experimental Procedures.** ○: control, 3-DU omitted; □: 0.5 μM 3-DU; △: 1.0 μM 3-DU; ◇: 2.0 μM 3-DU. Fig. 1 (Right panel): Effect of 3-DU on inhibition by ddC on the replication of an HIV-1 clinical isolate ($ERS104_{pre}$). Assays were performed as described in **Experimental Procedures.** ○: control, 3-DU omitted; ■: 0.1 μM 3-DU; □: 0.5 μM 3-DU; △: 1.0 μM 3-DU. Values shown are mean ± S.D. from quadruplicate determinations.

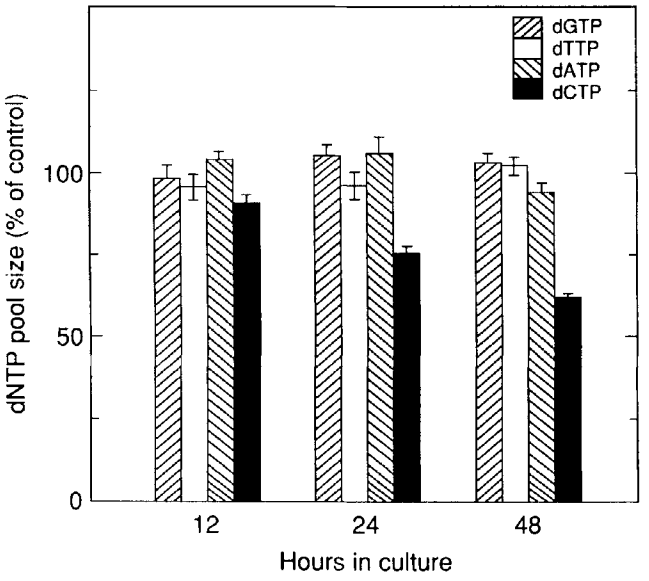

FIG. 2. Time-dependence of 3-DU effects on dNTP pools in PHA-stimulated PBM cells. Cells (5×10^7) were incubated in 20 ml of culture medium at 37°C, in the presence of 3-DU, 2 μM. At the indicated times, cell aliquots were harvested and cellular nucleotides extracted with 60% methanol as previously described.[12] Intracellular dNTP pools were quantified by an enzymatic assay.[13,14] dNTP pool sizes are expressed as percent of the pool sizes at t = 0. The dNTP values at t = 0 for dGTP, dTTP, dATP and dCTP were 1.0, 5.6, 1.2 and 2.1 pmol per 10^6 PHA-PBM cells respectively. Values represent the mean ± S.D. (n=3) from three donors, with duplicate determinations in each experiment.

TABLE 1

Effect of 3-DU on PHA-PBM cell replication.

PHA-stimulated PBM cells from normal subjects were incubated with increasing concentrations of 3-DU, each incubation being carried out in 20 ml of culture medium. Viable (trypan-blue excluding) cells were counted at 48 and 96 hr. Results are expressed as viable cells/ml of incubation medium. Cell count at t = 0: 55 x 10^4 cells/ml. Values shown are means ± S.D. from quadruplicate determinations.

3-DU	Cell number/ml (x 10^{-4})	
μM	48 hr	96 hr
0	93 ± 8	122 ± 9
0.01	96 ± 5	113 ± 7
0.10	91 ± 4	103 ± 6
1.0	86 ± 5	97 ± 4
10.0	60 ± 6	77 ± 3

TABLE 2

Effect of 3-DU on deoxycytidine kinase activity in PHA-PBM cells.

PHA-stimulated PBM cells from normal subjects were incubated for 20 hr with increasing concentrations of 3-DU, each incubation being carried out in 20 ml of culture medium. Deoxycytidine kinase activity was assayed as previously described.[12] Values shown are are means ± S.D. from quadruplicate determinations. Deoxycytidine kinase activity at t = 0 was 1.7 ± 0.1 nmoles dCMP generated/hr/mg protein.

3-DU	Deoxycytidine kinase activity
μM	(nmoles dCMP generated/hr/mg protein)
0	4.2 ± 0.3
0.5	5.0 ± 0.4
1.0	5.2 ± 0.4
2.0	5.8 ± 0.3

DISCUSSION

The ability of 3-DU (as its 5'-triphosphate) to inhibit CTP synthase is well known and has been characterized by several authors.[15,16] Since CDP (derived from CTP) is the substrate for the de novo production (via ribonucleotide reductase) of dCDP and thence of dCTP, 3-DU depletes not only CTP but its corresponding deoxynucleotide, dCTP (**FIG. 2**). In turn, since dCTP is a potent feedback inhibitor of deoxycytidine kinase,[17] the first enzyme in the deoxycytidine salvage pathway, a fall in dCTP pools will bring about a compensatory increase in the phosphorylation, not only of deoxycytidine, but also of the cytidine-based anti-HIV agents 3TC and ddC, alternate substrates for deoxycytidine kinase (an effect we previously observed with the 3-DU/ddC combination in a murine leukemia cell test system[18]). Thus the enhancement in the biological activity of these two agents in the presence of 3-DU-5'-triphosphate would appear to be a consequence of two effects of the latter drug: a fall in dCTP levels, leading to favorable shifts in the 3TCTP/dCTP and ddCTP/dCTP ratios, and an increase in the rate of phosphorylation of 3TC or ddC, also leading to favorable shifts in the relevant ratios. Although the increase in deoxycytidine kinase activity would also have the effect of increasing dCTP synthesis via the salvage pathway, the time-dependent decrease in total "net" dCTP pools after 3-DU exposure (**Fig. 2**) indicates that, in PHA-PBM cells, the 3-DU-induced inhibition in the de novo pathway for dCTP must be significantly greater than the countervailing stimulation of the salvage pathway, at least under the conditions of these experiments. Markedly absent is the many-fold increase in deoxycytidine kinase activity that we observed in a previous study with the ribonucleotide reductase inhibitor HU,[6] and that largely negated the use of the latter agent in the enhancement of cytidine-based ddNs. The increase in deoxycytidine kinase activity after 3-DU (1.0 μM) was only 24% (**TABLE 2**).

While use of a CTP synthase inhibitor such as 3-deazauridine is a convenient method for depleting dCTP, it is not an ideal agent for this purpose because of its indirect mode of action, i.e., inhibition of the production of the precursor ribotide CTP, leaving open the possibility of cytotoxicity arising from interference with host-cell RNA synthesis. Combination with a more specific inhibitor of dCTP synthesis would thus be desirable, permitting a decrease in the level of 3-DU required for potentiation. In an earlier study, we showed that thymidine (as its nucleotide dTTP), strongly potentiated ddC because of its ability to inhibit specifically the CDP-reductase activity of ribonucleotide reductase.[18] Since these two effects are independent of each other (i.e., while 3-deazauridine inhibits the CDP supply to ribonucleotide reductase, dTTP inhibits CDP's subsequent conversion to dCDP and thence to dCTP) it would appear likely that these two modes of dCTP pool reduction

could have an additive or even synergistic effect on the potentiation of cytidine-based anti-HIV agents such as ddC and 3TC. This possibility remains to be explored.

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