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Lamivudine (3TC) Phosphorylation and Drug Interactions *In Vitro*

*Stephen Kewn, Gareth J. Veal, Patrick G. Hoggard, Michael G. Barry and David J. Back**

Department of Pharmacology & Therapeutics, University of Liverpool, P.O. Box 147, Liverpool, L69 39E UK

ABSTRACT. Lamivudine (2′-deoxy-3′-thiacytidine; 3TC) is a dideoxynucleoside analogue that inhibits the replication of human immunodeficiency virus (HIV). We are currently investigating the intracellular metabolism of 3TC to its active triphosphate (3TCTP) in peripheral blood mononuclear cells (PBMC) and a monocytic cell line (U937). Optimal phosphorylation of 3TC was achieved after incubation for 24 hr, with 3TC diphosphate (3TCDP) the predominant metabolite formed, in both cell types investigated. Further studies in PBMCs followed preincubation with the mitogen phytohaemagglutinin (PHA) for 72 hr. This enabled greater detection of phosphates, compared to resting cells. A 3TC concentration of 1 μM was chosen for future interaction studies, allowing good detection of 3TC and phosphates on radiochromatograms whilst being similar to the plasma level found in clinical studies (i.e. 3 μM). With a shift in treatment to combination therapy, it is essential that potential interactions between nucleoside analogues are investigated at the phosphorylation level, as this could affect antiviral activity. Both deoxycytidine (dC) and 2′,3′-dideoxycytidine (ddC) significantly inhibited 3TC phosphorylation (e.g. at dC 100 μM, no 3TCTP was detected in PBMCs; $P < 0.001$, whereas 66% of control 3TCTP production was observed in U937 cells; $P < 0.01$). Zidovudine (ZDV) caused a small but significant reduction of 3TC phosphate production in both PBMCs and U937 cells. However, this may be due to toxicity or an effect on endogenous dCTP pools. Neither 2′,3′-dideoxyinosine (ddI) or 2′,3′-didehydro-2′,3′-dideoxythymidine (d4T) significantly inhibited 3TC phosphorylation. These results suggest it would be better to coadminister two nucleoside analogues with different activation pathways. BIOCHEM PHARMACOL **54**;5:589–595, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. 3TC; phosphorylation; combination therapy; drug interactions; *in vitro*; nucleoside analogues

3TC† is a dideoxynucleoside analogue that inhibits the replication of HIV [1, 2], the aetiologic agent of AIDS [3]. It is the negative isomer of a cytidine analogue where the 3′ carbon of the ribose ring has been replaced by a sulphur atom (Fig. 1). In contrast, the cytidine analogue ddC is a D-sugar-nucleoside. As with other dideoxynucleosides, 3TC must be metabolized to its respective 5′-triphosphate by intracellular kinases [4]. Nucleoside analogue triphosphates inhibit the HIV enzyme reverse transcriptase (RT), which catalyses the formation of complementary DNA from the single-stranded HIV RNA using host cell endogenous nucleosides as substrates. HIV replication is inhibited in two ways. Firstly, the active triphosphates compete with their corresponding endogenous nucleoside triphosphates for binding to RT. Once incorporated into viral DNA, chain termination results due to absence of a 3′-hydroxy group to which 3′-5′-phosphodiester linkages are normally made [5, 6].

Studies have shown that 3TC is generally less potent than ZDV and ddC, but more potent than ddI in inhibiting the replication of HIV *in vitro* [1]. Additionally, 3TC has been found to have a more favourable toxicity profile compared to other nucleoside analogues [7]. However, rapid high-level resistance to 3TC can arise via a mutation at codon 184 of RT [8]. This limits the use of 3TC as a monotherapy.

Results from the Delta [9] and ACTG 175 [10] clinical trials have shown a clear benefit of two nucleoside analogue drugs over monotherapy. The combination of ZDV with 3TC shows considerable promise. This is mainly due to reversal of ZDV resistance via the mutation at codon 184 of RT, conferring resistance to 3TC. The 184 mutation appears to effectively mask or suppress the effect of the ZDV resistance mutation *in vitro* [11]. *In vivo* studies have shown that ZDV/3TC combination therapy results in a

* Corresponding author: Professor D. J. Back, Department of Pharmacology & Therapeutics, University of Liverpool, P.O. Box 147, Liverpool. L69 39E UK. TEL: (0151) 794-5547, FAX: (0151) 794-5540, Email: daveback@liverpool.ac.uk.

† *Abbreviations:* AIDS, acquired immunodeficiency syndrome; CMP, cytidine monophosphate; dC, deoxycytidine; ddC, 2′,3′-dideoxycytidine; ddI, 2′,3′-dideoxyinosine; DP, diphosphate; d4T, 2′,3′-didehydro-2′,3′-dideoxythymidine; HIV, human immunodeficiency virus; MP, monophosphate; NDP, nucleoside diphosphate; NR, neutral red; PBMC, peripheral blood mononuclear cell; PHA, phytohaemagglutinin; PRPP, 5-phosphoribosyl-1-pyrophosphate; RT, reverse transcriptase; 3TC, 2′-deoxy-3′-thiacytidine, lamivudine; TP, triphosphate; and ZDV, zidovudine.

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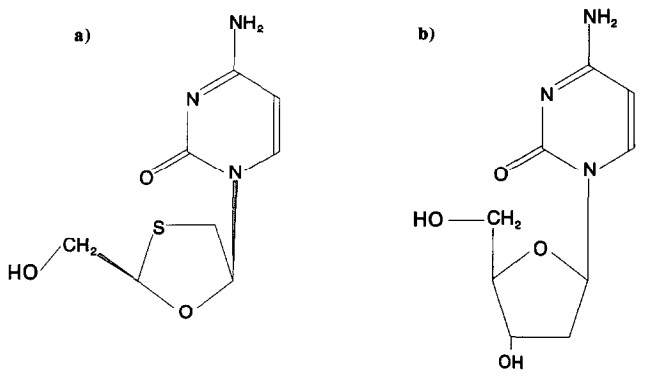

FIG. 1. Structures of (a) 3TC, and (b) the endogenous nucleoside dC.

markedly greater decrease in serum HIV-1 RNA concentrations than treatment with ZDV alone, even though codon 184 mutants rapidly emerged [12].

With a shift in treatment of HIV to combination therapy, it is essential that potential interactions of drugs or metabolites are investigated for any changes in the phosphorylation of the nucleoside analogues, which in turn, will affect antiviral activity. We have previously shown the effect of various drugs on ZDV, d4T and ddC phosphorylation *in vitro* [13–17]. In the present work we have evaluated the effects of the nucleoside analogues (ZDV, ddC, ddI, d4T) and the endogenous nucleoside, dC, on 3TC phosphorylation *in vitro*. These investigations were performed in peripheral blood mononuclear cells (PBMC) and U937 cells (a monocytic cell line). PBMCs were preincubated with PHA, which has been shown to stimulate intracellular kinase activities [18].

MATERIALS AND METHODS

Materials

CHEMICALS. Lymphoprep was purchased from Nycomed Pharma AS, Oslo, Norway. Foetal calf serum was acquired from Sera Lab, Sussex. UK. 3TC, [^{3}H]3TC (Specific activity—12.2 Ci mmol^{-1}) and 3TCTP were generously donated by Glaxo-Wellcome, UK. ddI and d4T were gifts from Bristol-Myers Squibb, Wallingford, CT. ddC was a gift from Roche products Ltd, Welwyn Garden City, UK. All other drugs and chemicals were purchased from Sigma Chemical Company Ltd., UK.

Intracellular Phosphorylation of 3TC by Isolated PBMCs

ISOLATION OF PBMCs. Fresh heparinised venous blood (10 mL) from different healthy volunteers was carefully layered onto lymphoprep resolving medium (5 mL), centrifuged (693 × g; 22 min; 4°C) and the central band corresponding to mononuclear cells removed and added to fresh RPMI medium. The resulting cell suspension was centrifuged (693 × g; 5 min; 4°C), the supernatant fraction discarded and the cell pellet resuspended in RPMI media (supplemented with foetal calf serum [10%], L-glutamine [2 mM], and penicillin [5000 units/mL]/streptomycin [5000 μg/mL]).

STIMULATION OF CELLS WITH MITOGEN. Cells were seeded in 5 cm diameter petri dishes (3×10^6 cells plate^{-1}). The mitogen, PHA (5 μg ml^{-1}) was added and the total volume was made up to 4 mL with RPMI growth media. Cells were cultured at 37°C in a humidified 5% CO_2 gassed incubator for 72 hr.

INCUBATION OF PBMCs WITH [^{3}H]3TC. To optimise assay conditions, 3TC phosphorylation was assessed in both resting and PHA-activated PBMCs. The effect of increasing incubation time (from 3–36 hr), and the effect of increasing 3TC concentration (from a base level of 0.013 μM to 0.1, 1 and 10 μM—a 770-fold increase) was investigated in stimulated PBMCs incubated for 24 hr.

Drug interaction studies involved preincubation of PBMCs with PHA for 72 hr. Cells were then incubated with [^{3}H]3TC (0.65 μCi; 1 μM) for 24 hr in a humidified, 5% CO_2 gassed incubator. The effects of ZDV, ddI, ddC, d4T, and dC on phosphorylation were studied by addition of 40 μL of 10 μM, 100 μM, 1 mM, and 10 mM solutions to each plate to give final drug concentrations of 0.1, 1, 10, and 100 μM, respectively. To control plates additional growth media was added. Experiments were performed in triplicate on at least four separate occasions.

CELL COLLECTION AND EXTRACTION. Petri dishes were scraped clean of cells and the cell suspensions where transferred to clean 7 mL tubes, centrifuged (2772 × g; 4 min; 4°C) and the supernatant fraction discarded. The resulting cell pellets were washed with 500 μL PBS, recentrifuged (2772 × g; 4 min; 4°C) and the supernatant fraction removed. Five hundred microliters of 60% methanol was then added to the cells, which were vortexed and extracted overnight at 4°C. After extraction, samples were centrifuged to remove cellular debris and the methanol extracts of each set of plates pooled. A 50 μL aliquot of the extract was counted to determine the total intracellular radioactivity. The 60% methanol was then evaporated under a steam of nitrogen. Samples were stored at −20°C until HPLC analysis.

U937 CELL INCUBATIONS. U937 cells are derived from monocytes. Cells were routinely maintained in 75 cm^2 flasks containing RPMI medium supplemented with 10% foetal calf serum and L-glutamine (2 mM) at 37°C in a humidified, 5% CO_2 gassed incubator.

To optimise assay conditions, studies similar to those in PBMCs were performed with respect to incubation time and 3TC concentration.

For drug interaction studies U937 cells (4×10^6) were incubated with [^{3}H]3TC (0.65 μCi; 1 μM) and a test drug over a range of concentrations (0.1–100 μM) for 24 hr at 37°C in a humidified, 5% CO_2 gassed incubator. Experi-

ments were performed in triplicate in six-well multiplates on at least four separate occasions. Following incubation, cell suspensions were transferred to 7 mL silanised tubes, centrifuged, washed, recentrifuged, and extracted as described earlier for stimulated PBMCs.

HPLC ANALYSIS. Cell extracts were reconstituted in 50 μL of double distilled water prior to HPLC analysis. 3TC and its phosphate metabolites were separated by HPLC on an anion exchange column (Partisil 10-SAX; 25 cm × 4.6 mm), eluted with a gradient from 1 mM potassium dihydrogen orthophosphate (pH 4.5; Buffer A) to 500 mM potassium dihydrogen orthophosphate/800 mM potassium chloride (pH 3.5; Buffer B).

The gradient ran between 0 and 8 min with Buffer A at a flow rate of 1.0 ml/min (which remains constant throughout the run). Between 8 and 28 min, the linear gradient changed from 100% Buffer A to 100% Buffer B and at 28 min to 33 min Buffer B ran isocratically. A linear gradient of 100% Buffer B to 100% Buffer A was run between 33 and 43 min. Buffer A then ran from 43 to 50 min. From 50.1 to 55 min the column was washed through with double distilled water and then reequilibrated to 100% Buffer A, which ran from 55.1 to 60 min [16]. 3TC and 3TCTP were identified by cochromatography with authentic standards. 3TCMP and 3TCDP were assigned according to their elution order by cochromatography with standards obtained by hydrolysis of the 3TCTP standard. The peaks were also identified as phosphates via hydrolysis with acid phosphatase (0.5 mg mL^{-1}) for 2 hr at 37°C.

Cell Viability

NEUTRAL RED ASSAY. Cell viability in the presence of interacting drugs was assessed by the method of Neutral red (NR) uptake. NR dye (50 μg mL^{-1}) was added for a period of 3 hr, prior to termination of the incubation. Cells were harvested as described followed by two washes with phosphate buffered saline (500 μL). Destaining solution (1% glacial acetic acid, 50% ethanol; 500 μL) was then added to release the dye taken up. After thorough mixing the absorbance of the homogenous solutions was measured at 550 nm on a scanning multiwell spectrophotometer.

Statistical Methods

Phosphorylation and NR data were analysed by analysis of variance (ANOVA) followed by a modified *t*-test (Bonferroni).

RESULTS

3TC (0.65 μCi; 0.013 μM) was phosphorylated in resting PBMCs (total phosphates 0.070 ± 0.059 pmol/10^6 cells; $n = 4$), and to a greater extent in PHA-stimulated PBMCs (total phosphates 0.512 ± 0.082 pmol/10^6 cells; $n = 4$; $P < 0.001$). Additional studies investigated the effect of incubation time on 3TC phosphorylation. An incubation time of 24 hr was chosen for future studies, as this time resulted in the greatest phosphate production in both cell types.

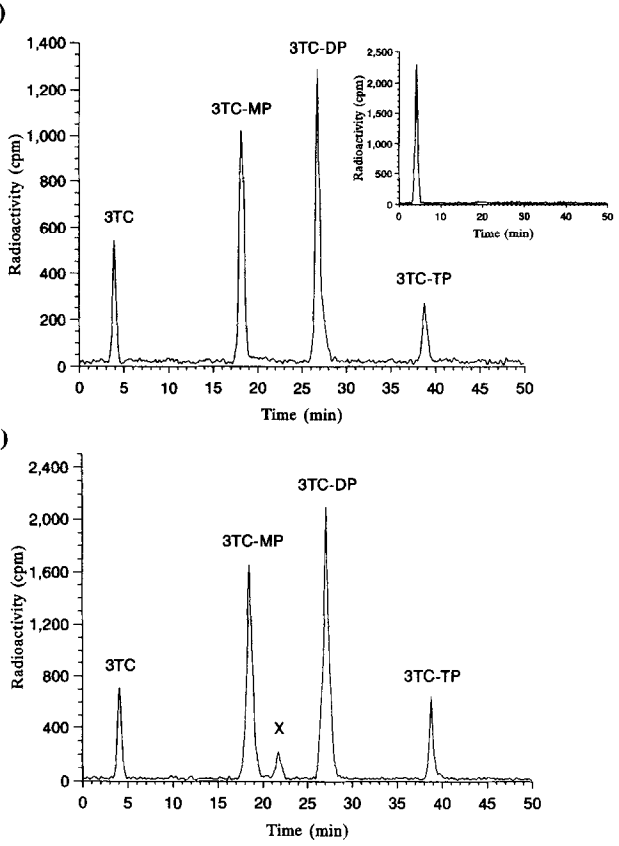

FIG. 2. Radiochromatograms showing detection of [^{3}H]3TC and phosphorylated metabolites after 24 hr incubations with 3TC (0.65 μCi; 1 μM) in (a) PHA-stimulated PBMCs and (b) U937 cells. Inset radiochromatogram shows PBMC sample after treatment with acid phosphatase (0.5 mgml^{-1}, 37°C, 2 hr).

Typical HPLC profiles for the production of 3TC phosphates in PHA-stimulated PBMCs and U937 cells, following incubation of 3TC (0.65 μCi; 1 μM) for 24 hr, are shown in Fig. 2. The profiles are similar, with 3TCDP the predominant metabolite (50% of total phosphates in PHA-stimulated PBMCs, 45% in U937 cells). 3TCMP and 3TCTP accounted for 35 and 15% of total phosphates, respectively, in both PHA-stimulated PBMCs and U937 cells. An additional metabolite (X) was consistently observed in U937 cells but rarely in PHA-stimulated PBMCs.

The effect of increasing the extracellular 3TC concentration on phosphorylation is illustrated in Fig. 3. Increasing the extracellular 3TC concentration from a base level of 0.013 μM, to 0.1, 1, and 10 μM (in PHA-stimulated PBMCs) led to clear evidence of saturation of 3TCTP formation, e.g. at 10 μM there was a 900-fold increase in 3TC levels but only a 100-fold increase in 3TCTP levels. Assuming that a eukaryotic cell has a volume of 1pl [4], the intracellular triphosphate concentrations were 0.10, 0.35, 2.29, and 7.84 μM, respectively. A similar pattern was

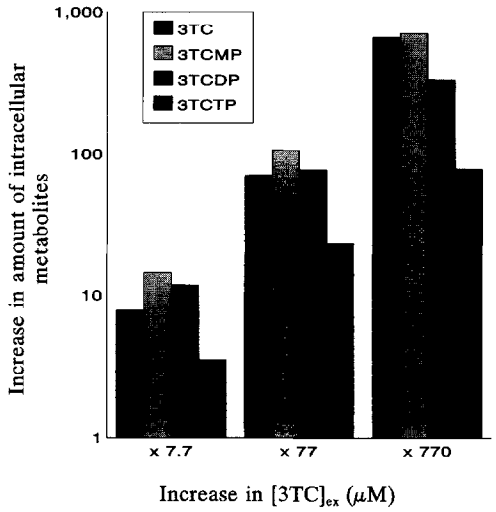

FIG. 3. Effect of increasing extracellular 3TC concentration ($[3TC]_{ex}$) from the initial concentration of 0.013 μM to 0.1, 1, and 10 μM (770-fold), on the detection of individual 3TC metabolites in PHA-stimulated PBMCs. Results show mean values from $n = 5$ following a 24 hr incubation.

observed in U937 cells. A concentration of 1 μM was chosen for future interaction studies in both PHA-stimulated PBMCs and U937 cells. This is similar to the clinical plasma concentration (around 3 μM), while still enabling good detection of 3TC radiochromatograms *in vitro* [19].

The endogenous nucleoside dC significantly inhibited the formation of 3TC phosphates in PHA-stimulated PBMCs at both 10 μM (42% of control total phosphates; $P < 0.001$; Fig. 4a, with no 3TCTP detected; $P < 0.001$; Fig. 4b) and 100 μM (6% of control total phosphates; $P < 0.001$; Fig. 4a, with no 3TCTP detected; $P < 0.001$; Fig. 4b). 3TC phosphorylation was also reduced by dC, but to a

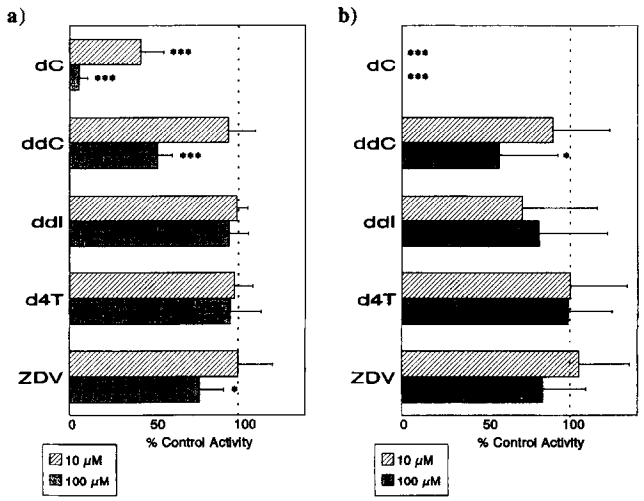

FIG. 4. Effect of dC and the nucleoside analogues on (a) total 3TC phosphorylation and (b) individual 3TCTP production, in PHA-stimulated PBMCs. Each bar represents mean ± SD $n = 5$ ($n = 4$ for dC). Data analysed by ANOVA followed by modified *t*-test (Bonferroni), $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. No 3TCTP was detected at dC 10 or 100 μM.

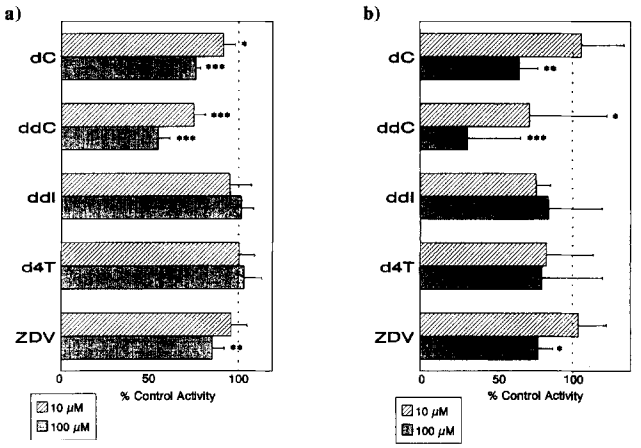

FIG. 5. Effect of dC and the nucleoside analogues on (a) total 3TC phosphorylation and (b) individual 3TCTP production, in U937 cells. Each bar represents mean ± SD $n = 5$ ($n = 4$ for dC). Data analysed by ANOVA followed by modified *t*-test (Bonferroni), $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

much lesser extent, in U937 cells at concentrations of 10 μM (92% of control total phosphates; $P < 0.05$; Fig. 5a, with no effect on 3TCTP production; Fig. 5b) and 100 μM (77% of control total phosphates; $P < 0.001$; Fig. 5a, 66% of control 3TCTP production; $P < 0.01$; Fig. 5b).

The nucleoside analogue ddC significantly decreased 3TC phosphorylation in PHA-stimulated PBMCs at a concentration of 100 μM (58% of control 3TCTP production; $P < 0.05$; Fig. 4b). ddC reduced 3TC phosphate production in U937 cells at 10 μM (73% of control 3TCTP production; $P < 0.05$) and 100 μM (32% of control 3TCTP production; $P < 0.001$) (Fig. 5b). However, some toxicity (as determined by the NR assay) was observed in U937 cells incubated with 100 μM ddC for 24 hr (90% of control NR uptake; Table 1). Therefore, this study was repeated with a 5 hr incubation to minimize toxicity. There was a significant reduction in 3TC phosphates at 100 μM ddC (29% of control 3TCTP production; $P < 0.001$; Table 1), without any change in NR uptake.

ZDV, at a concentration of 100 μM, reduced levels of 3TCTP in PHA-stimulated PBMCs (84% of control 3TCTP production; Fig. 4b), but this failed to reach significance. However, 3TCTP levels were significantly decreased in U937 cells at this concentration (78% of control 3TCTP production; $P < 0.05$; Fig. 5b). In contrast, d4T, a thymidine analogue similar to ZDV, had no inhibitory effect on 3TC phosphorylation in either PHA-stimulated PBMCs or U937 cells (Figs. 4 and 5). There was also no significant inhibition of 3TC phosphate production observed with ddI.

DISCUSSION

3TC is a nucleoside analogue that has promising anti-HIV activity in combination with other dideoxynucleosides, with little or no toxicity. Although the enzymes responsible for the phosphorylation of 3TC to its active triphosphate

TABLE 1. The effect of ddC on 3TC phosphorylation in U937 cells

Conditions	pmol/10^6 cells					% Control	
	3TC	3TC-MP	3TC-DP	3TC-TP	Total phosphates	Total phosphates	Neutral red uptake
5 h Incubation							
Control	3.68 ± 0.49	1.66 ± 0.39	4.23 ± 0.24	3.80 ± 1.95	9.69 ± 2.28	—	—
ddC 1.0 μM	3.64 ± 0.53	2.03 ± 0.18	4.58 ± 0.42	3.32 ± 2.31	9.93 ± 2.29	102.9 ± 10.0	100.3 ± 7.1
ddC 10.0 μM	3.74 ± 0.86	1.93 ± 0.36	3.73 ± 0.87	3.29 ± 1.79	8.95 ± 2.14	92.3 ± 1.7	102.9 ± 8.0
ddC 100.0 μM	3.46 ± 0.31	1.86 ± 0.52	3.37 ± 0.73*	1.01 ± 0.79***	6.24 ± 1.36***	64.7 ± 3.4	104.1 ± 7.5
24 h Incubation							
Control	4.13 ± 1.49	9.03 ± 2.12	12.72 ± 1.57	3.81 ± 2.04	25.56 ± 1.93	—	—
ddC 1.0 μM	4.57 ± 2.76	7.69 ± 2.43*	12.93 ± 2.00	3.12 ± 1.79	23.74 ± 2.42**	92.9 ± 6.4	97.7 ± 8.5
ddC 10.0 μM	4.60 ± 2.51	7.40 ± 2.66**	9.04 ± 2.21***	2.79 ± 2.33*	19.22 ± 2.17***	75.3 ± 6.9	87.9 ± 12.2
ddC 100.0 μM	3.91 ± 0.68	6.44 ± 1.94***	6.17 ± 2.15***	1.39 ± 1.72***	14.01 ± 1.95***	54.9 ± 6.9	90.6 ± 14.8

Results expressed as the mean ± SD $n = 4$ (5 h), $n = 5$ (24 h) for 3TC interaction; $n = 3$ (5 h), $n = 4$ (24 h) for toxicity experiments. Data analyzed by modified t-test, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

have yet to be fully elucidated, it is thought that this activation is mediated by the same enzymes involved in the metabolism of ddC (Fig. 6). This is supported by studies showing that the initial conversion of 3TC to 3TCMP is mediated by deoxycytidine kinase [20].

Previous studies have demonstrated a twofold increase in deoxycytidine kinase activity brought about by mitogen stimulation in PBMCs [18]. However, levels of 3TC phosphates increased more than sixfold in PBMCs stimulated with PHA for 72 hr, compared to resting PBMCs, after a 24 hr incubation. Additionally, Gao *et al.* [21] found that intracellular 3TCTP levels were lower in resting PBMCs than in PHA-stimulated PBMCs, supporting these findings. Therefore, it was decided that future studies in PBMCs would involve preincubation with PHA for 72 hr.

Phosphorylation of 3TC to its three major anabolites was similar in PHA-stimulated PBMCs and U937 cells (Fig. 2). The HPLC profiles show accumulation of 3TCDP, suggesting that conversion to 3TCTP may be the rate-limiting step. This contrasts with ddC phosphorylation where no predominant metabolite was observed [16]. An additional metabolite (X) was detected almost exclusively in U937 cells. Although this has yet to be rigorously identified, X may be postulated to be 3TCDP choline based on similar

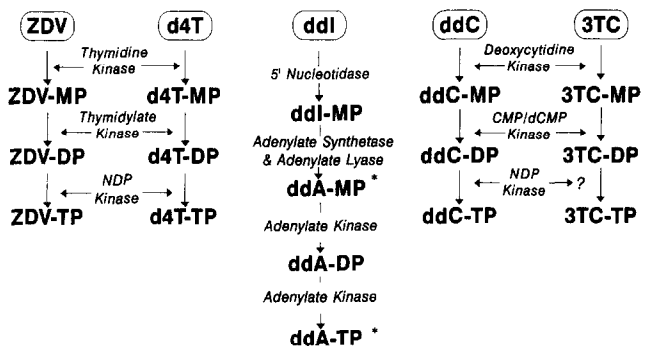

FIG. 6. Activation pathways of some clinically important nucleoside analogues. (NDP—nucleoside diphosphate; CMP—cytidine monophosphate). *There is also the possible involvement of PRPP synthetase in the formation of ddA-TP from ddA-MP.

studies with ddC phosphorylation [16]. Maximal phosphorylation of 3TC was achieved after incubation for 24 hr in both cell types investigated.

When the extracellular concentration of 3TC was increased to a clinically relevant level in PHA-stimulated PBMCs, there was a nonlinear increase in intracellular 3TCDP and 3TCTP levels (Fig. 3). Similar results were obtained for U937 cells. Because the increase in intracellular parent 3TC levels was greater than that observed for 3TCDP and 3TCTP, it indicates that an activation step has become saturated, principally for conversion to 3TCTP. In contrast, studies performed by Gray *et al.* [22] using PHA-stimulated peripheral blood lymphocytes (PBL), found that the level of intracellular 3TCTP was linearly dependant on the extracellular 3TC concentration (between 0.1 and 10 μM) after a 4 hr incubation. However, these differences may be explained by a shorter incubation time adopted by these workers. Although earlier studies of intracellular 3TC phosphorylation in PBLs showed no substantial difference in the formation of 3TCTP over time, these data were obtained at an extracellular 3TC concentration of 10 μM [23]. At concentrations above 10 μM, Gray *et al.* found saturation of the enzyme responsible for conversion to 3TCTP, suggesting at 10 μM the level of 3TCTP would not change over time.

The effects of ddC, ZDV, d4T, ddI, and the endogenous nucleoside dC on 3TC phosphorylation were investigated in PHA-stimulated PBMCs and U937 cells over 24-hr incubation periods. This ensured optimal turnover of 3TC to its phosphates, enabling accurate determination of potential interactions. The range of concentrations at which the interacting drugs were studied (0.1–100 μM) covered their clinically relevant plasma levels [24–27]. Because 3TCTP is thought to be solely responsible for the antiviral activity of 3TC, measurement of potential drug interactions was determined by changes in 3TCTP production as well as total phosphate formation.

dC and ddC decreased 3TC phosphorylation in both PHA-stimulated PBMCs and U937 cells (Figs. 4 and 5).

This is probably because ddC, 3TC, and dC are all substrates for the enzyme responsible for initial conversion to 3TCMP, deoxycytidine kinase [20, 28]. Differences were observed with the interaction of dC and 3TC, with a greater inhibitory effect found in PHA-stimulated PBMCs compared to U937 cells. This could suggest that the affinities of dC and/or 3TC for deoxycytidine kinase vary in these cell types. Alternatively, differences in deamination levels of dC have been observed by other workers, which could also account for these findings [29].

Studies performed by Veal *et al.* [16] investigating ddC phosphorylation, found that 3TC inhibited ddC phosphorylation in a concentration-dependent manner at identical concentration ratios (10 and 100). However, the level of inhibition was much greater compared to the effect of ddC on 3TC phosphorylation. These data can be supported by studies carried out by Shewach *et al.* [30] in Molt-4 T lymphoblasts. They found that 3TC was a more efficient substrate for deoxycytidine kinase than ddC (V_{max}/K_m for 3TC 0.41 and 0.66; V_{max}/K_m for ddC 0.15 and 0.22, with ATP and UTP as phosphate donors, respectively). From a clinical perspective this interaction could be very important, as 3TC plasma levels are much greater than those for ddC (around 3 μM, compared to 0.1 μM). Therefore, while ddC inhibits 3TC phosphorylation at high concentration ratios (10 and 100), it is unlikely that this situation would arise clinically. In contrast, ddC phosphorylation is reduced by 3TC at similar concentration ratios to those observed in patients given the two drugs. Hence, the use of 3TC and ddC in combination may not give optimal benefit to the patient.

ZDV, at a high concentration (100 μM), was also seen to inhibit 3TC phosphorylation (Figs. 4 and 5). There are three possible explanations. Firstly, ZDV may compete with 3TC for the enzyme thought to be responsible for the conversion from DP to TP (of both drugs), NDP kinase. However, this does not explain why 3TCDP levels were the most predominantly affected.

Secondly, ZDV may be toxic to PBMCs and U937 cells. Although no toxicity was noted, as determined by the NR assay (in U937 cells), ZDV-mediated mitochondrial toxicity has been observed by other workers [31, 32]. Finally, ZDV may influence dCTP pools, which in turn, could affect 3TC phosphorylation. Frick *et al.* [33] showed a rise of dCTP pools in cells treated with ZDV, which could explain the effects on 3TC phosphorylation observed.

Compared to ZDV, the thymidine analogue d4T had no effect on 3TC phosphorylation in PHA-stimulated PBMCs or U937 cells (Figs. 4 and 5). The activation of d4T is mediated by the same enzymes as ZDV [34]. Therefore, the data suggest competition for activation to the TP is not responsible for the interaction observed between ZDV and 3TC. d4T has a much more favourable toxicity profile compared to ZDV [35], which supports the hypothesis that the interaction observed between 3TC and ZDV is due to toxicity, and not competition for certain activation steps. However, it is also known that unlike ZDV, d4T has no effect on dCTP pools [21]. This is a more likely explanation of the interactions observed.

ddI also had no effect on 3TC phosphorylation (Figs. 4 and 5) due to it sharing none of the enzymes involved in the activation of both drugs (Fig. 6) [36].

These *in vitro* studies are important when considering which drugs should be used in combination therapy in clinical trials. The phosphorylation data suggest it is better to coadminister two nucleoside analogues with different activation pathways, such as 3TC and ZDV, rather than those with shared enzymes, e.g. 3TC and ddC, to ensure maximal anti-HIV activity.

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