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CELLULAR METABOLISM OF (−) ENANTIOMERIC 2′-DEOXY-3′-THIACYTIDINE

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Abstract—The metabolism of (−) enantiomeric 2′-deoxy-3′-thiacytidine (3TC) was examined in human immunodeficiency virus type 1 (HIV-1)-infected and mock-infected human cells. 3TC 5′-triphosphate levels accumulated comparably in HIV-1-infected and mock-infected phytohaemagglutinin-stimulated peripheral blood lymphocytes (PBL) and reached 40% or more of total intracellular 3TC metabolites after 4 hr. The rate of decay of 3TC triphosphate in HIV-1-infected and mock-infected PBL measured as a half-life ($T_{1/2}$) ranged from 10.5 to 15.5 hr. 3TC did not significantly affect metabolism of deoxynucleotides in the U937 cell line, and was shown to be resistant to the action of human platelet pyrimidine nucleoside phosphorylase.

A number of purine and pyrimidine nucleoside analogues without hydroxyl groups at the 2′ and 3′ positions inhibit infection of cells *in vitro* by the aetiological agent of acquired immune deficiency syndrome, human immunodeficiency virus (HIV†) [1]. Nucleosides such as 3′-azido-3′-deoxythymidine (AZT), 2′,3′-dideoxycytidine (ddC) and 2′,3′-dideoxyinosine appear to exert their antiviral activity on the virus encoded reverse transcriptase after metabolism by the cell to their 5′-triphosphate derivatives [2].

3TC, the (−) enantiomer of 2′-deoxy-3′-thiacytidine, is an effective inhibitor of HIV type 1 (HIV-1) and HIV-2 replication *in vitro* acting at an early stage in the virus life cycle [3]. In common with other nucleoside analogues the 5′-triphosphate derivative of 3TC inhibits HIV-1 reverse transcriptase *in vitro* and also acts as a chain terminator [4].

In this report, the intracellular metabolism of 3TC was examined in HIV-1-infected and mock-infected human peripheral blood lymphocytes (PBL) *in vitro*. The effects of 3TC and its metabolites on normal nucleotide metabolism was also determined by measuring intracellular deoxynucleotide pools in U937 cells, a cell line of monocyte–macrophage lineage [5].

Some pyrimidine nucleoside analogues have been shown to be effective substrates for thymidine phosphorylase isolated from human blood platelets [6]. The degradation of novel nucleoside analogues by phosphorolytic cleavage may reduce their therapeutic efficacy, and may increase the probability of formation of toxic metabolites. The catabolism of 3TC by human platelet phosphorylase was therefore investigated.

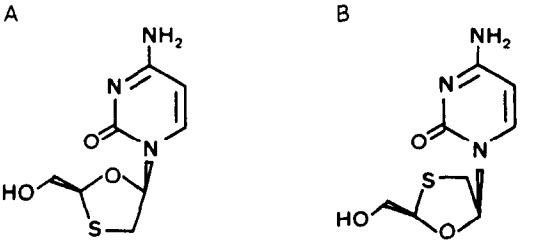

Fig. 1. Structure of 2′-deoxy-3′-thiacytidine. (A) (+) enantiomer, (B) (−) enantiomer (3TC).

MATERIALS AND METHODS

Materials. Racemic (+/−) 2′-deoxy-3′-thiacytidine (BCH189) was synthesized at Glaxo Group Research Ltd and separated by chiral HPLC into the (−) enantiomer (3TC) and the (+) enantiomer. The chemical structures are shown in Fig. 1.

3TC 5′-monophosphate and 5′-triphosphate derivatives were also synthesized at Glaxo Group Research Ltd. Thymidine, thymine, deoxythymidine, uracil, cytidine, cytosine and AZT were purchased from the Sigma Chemical Co. (Poole, U.K.). All other reagents were of the highest grade available.

Radiochemicals. Racemic [^{3}H]2′-deoxy-3′-thiacytidine (BCH189) was purchased from Moravek (Brea, CA, U.S.A.) and separated by chiral HPLC into the enantiomeric forms. The (−) and (+) enantiomers had specific activities of 21.8 and 22.2 Ci/mmol, respectively.

Isolation and infection of human PBL. PBL obtained from HIV-1 negative donors were isolated by density gradient centrifugation through Ficoll lymphocyte separation medium (Flow Laboratories,

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† Abbreviations: 3TC, (−) enantiomeric 2′-deoxy-3′-thiacytidine; AZT, 3′-azido-3′-deoxythymidine; ddC, 2′,3′-dideoxycytidine; PBL, peripheral blood lymphocyte(s); PHA, phytohaemagglutinin; HIV, human immunodeficiency virus.

Irvine, U.K.) and activated with 5 μg/mL phytohaemagglutinin (PHA) (Boehringer Mannheim, Lewis, U.K.) for 48 hr. Stimulated PBL were washed once with RPMI medium containing 10% foetal calf serum, 100 IU/mL penicillin, 100 μg/mL streptomycin, 0.55 mM L-glutamine, 0.075% sodium bicarbonate and 1 × non-essential amino acids (all reagents from Flow Laboratories) (RPMI growth medium), and infected with HIV-1 strain RF at a multiplicity of infection of 1×10^{-2} tissue culture infectious doses/cell or mock infected for 60 min at room temperature. The PBL were washed twice with RPMI growth medium and incubated in the presence or absence of radiolabelled compound as described below.

Intracellular phosphorylation of [^{3}H]3*TC in HIV-1-infected and mock-infected PBL.* Samples of 2×10^6 cells were incubated with 10 μM [^{3}H]3TC at 37° in 5% CO_2 for up to 24 hr. After each time period, the cells were washed in RPMI growth medium and extracted with 80 μL 0.5 M cold perchloric acid (BDH, Poole, U.K.) for 60 min at 4°.

The supernatant was cleared at 12,000 *g* for 5 min and stored at −20°. Samples of 40 μL (corresponding to 1×10^6 cells) were injected into a Zorbax 10 SAX HPLC column (Anachem, Luton, U.K.).

At a flow rate of 1 mL/min, 5′-phosphorylated products were separated through a gradient system using 10 mM KH_2PO_4 pH 3.5 (mobile phase A) and 1 M KH_2PO_4 pH 3.5 (mobile phase B). The separation procedure consisted of a linear gradient of 0–100% B from 0 to 7 min, followed by isocratic 100% B to 17 min and a linear gradient to 100% A from 17 to 20 min. UV absorbance was detected at 262 nm. Radioactivity was monitored by running FloScint IV scintillation through an A-250 radiomatic detector (Canberra Packard, Pangbourne, U.K.) at a ratio of 4:1. Total flow rate was 5 mL/min. Profiles were automatically adjusted for quench effects using an internal standard run.

Intracellular decay of [^{3}H]3*TC triphosphate.* Samples of 2×10^6 cells were incubated for 4 hr with 10 μM [^{3}H]3TC or corresponding (+) enantiomer at 37° in 5% CO_2. The cells were washed three times and resuspended in RPMI growth medium. Samples of 2×10^6 cells at 0, 4, 19 and 24 hr were extracted with 80 μL of 0.5 M cold perchloric acid for 60 min at 4°. The supernatant was clarified at 12,000 *g* for 5 min and stored at −40°. Samples of 40 μL (corresponding to 1×10^6 cells) were injected into the HPLC column and the profiles of dpm and UV recorded on the A-250 radiomatic detector (Canberra Packard).

The effect of 3*TC on deoxynucleotide pools.* Cells were seeded at 1×10^5 cells/mL in 1-L stirred flasks (Techne (Cambridge), Duxford, Cambridge, U.K.), gassed for 10 seconds with CO_2, sealed and stirred at 37°. After 24 hr, test compounds dissolved in RPMI growth medium were added. After a further 12 hr at 37°, cells were collected and washed twice with 10 mL of cold phosphate-buffered saline containing 0.1% glucose. Pellets of 10^9 cells were resuspended in 1 mL phosphate-buffered saline. Cold trichloroacetic acid was added to produce 0.3 M final concentration and the suspensions left for 30 min on ice. Samples were centrifuged for 20 sec at 15,600 *g* and the supernatants added to 1.1 volumes of cold Freon containing 0.5 M tris-*N*-octylamine. Samples were mixed and centrifuged briefly at 15,600 *g*. The upper aqueous layer contains extracted nucleotides.

Ribonucleotides were removed from cell extracts by a method adapted from a previously published procedure [7]. Twenty microliters of 20 mM deoxyguanosine, to prevent dGTP oxidation, and 20 μL of 200 mM sodium periodate were mixed with 80 μL nucleotide extract. After 2 min at 37°, 2 μL of 1 M rhamnose and 30 μL of 4 M methylamine pH 6.5 were added. After a further 30 min at 37°, the samples were cooled on ice and analysed on a Partisil 10 SAX column, 4.6 × 250 mm (Whatman Lab. Sales, Maidstone, U.K.), and a solvent gradient of 0.2–1 M $NH_4H_2PO_4$, pH 4.0, containing 7% ethanol. Peaks were detected by UV absorption at 254 nm with a model 484 detector (Waters, Watford, U.K.) and integrated with Waters Maxima 820 software.

The effect of human platelet nucleoside phosphorylase on 3*TC.* EDTA-anticoagulated fresh human blood (50 mL) was centrifuged at 2500 rpm for 10 min at 4°. The platelet-rich supernatant was centrifuged at 5000 *g* for 10 min at 4° and the pellet washed three times and resuspended with buffer containing 20 mM Tris–HCl pH 7.4, 0.15 M NaCl, 1 mM EDTA, and re-centrifuged. The platelets were then resuspended in 5 mL of incubation buffer which contained 50 mM KH_2PO_4, 18 mM Tris–HCl pH 7.4, 0.135 M NaCl and 0.9 mM EDTA.

Test compounds (thymidine, deoxyuridine, cytidine, BCH189, (+) and (−) enantiomers) were added to platelet suspension at a final concentration of 50 μg/mL and incubated at 37° with frequent shaking. After various incubation times, 100-μL aliquots of the mixtures were cooled rapidly, centrifuged for 2 min at maximum speed in a MSE microcentrifuge (Fisons Instruments, Crawley, U.K.) and stored at −20°. Samples of 10 μL were analysed by HPLC on a reverse phase column (Zorbax ODS 4.6 × 150 mm, Anachem) with 0.01–0.1 M KH_2PO_4 pH 5.0 as mobile phase and a flow rate of 0.5–1 mL/min depending on the samples. Peaks were detected by UV absorption at the λ max of the compounds with a photodiode array detector (Waters), and identified by comparing the retention times and the absorption spectra of appropriate standards. Integrated peak areas were used to calculate the percentage of substrate and product(s).

RESULTS

Identification of phosphorylated derivatives of [^{3}H]3*TC*

A representative radiochromatogram of soluble [^{3}H]3TC cellular metabolites produced in PHA-stimulated, mock-infected PBL after 4 hr incubation is shown in Fig. 2. A similar profile was obtained for the (+) enantiomer (data not shown). Peaks numbered 1, 2 and 4 in Fig. 2 were identified as parent, monophosphate and triphosphate derivatives, respectively, based on comparison with non-radioactive standards run with the samples. Peak 3 is identified tentatively as 3TC diphosphate based

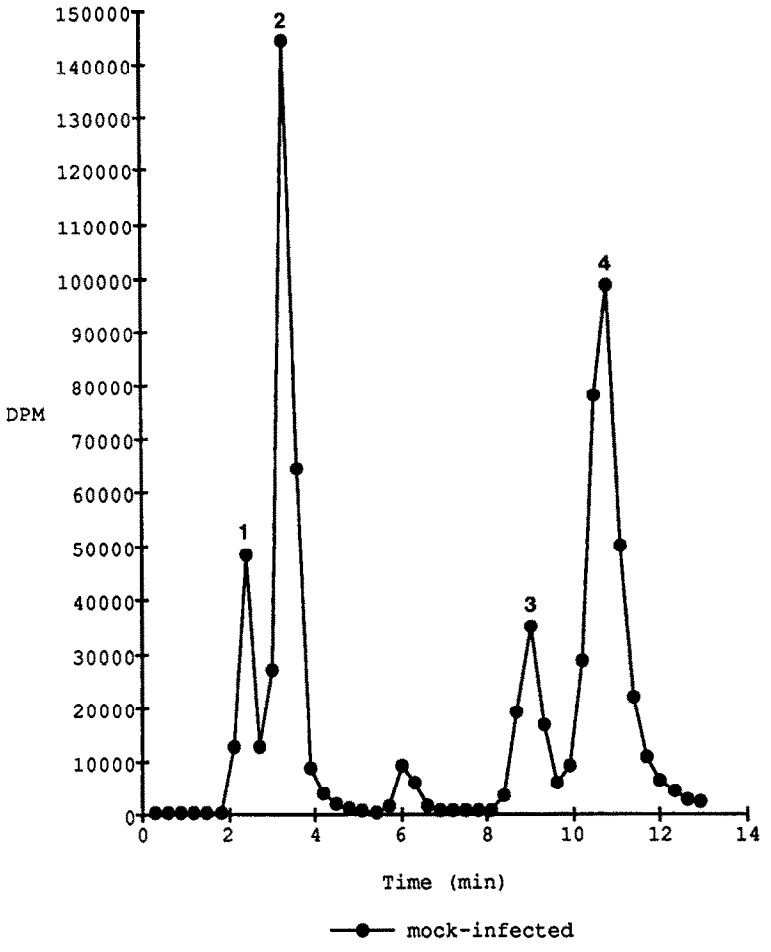


Fig. 2. Representative radiochromatogram of soluble [^{3}H]3TC cellular metabolites from PHA-stimulated human PBL cultures. Radiochromatogram of mock-infected PBL from donor C is plotted as dpm against time (min). By comparison with non-radioactive standards detected by UV absorbance, parent (peak 1), monophosphate (peak 2) and triphosphate (peak 4) were identified. Peak 3 is provisionally identified as diphosphate (no standard available).

Table 1. Peak summaries of soluble [^{3}H]3TC cellular metabolites from PHA-stimulated human PBL cultures

Peak No.	Retention time (min)	Integrated area (dpm)	% of total radioactivity
1	2.2	129744	18.39
2	3.2	72292	10.25
3	8.9	163759	23.22
4	10.6	339575	48.14

on its relationship to the positions of analogous 3TC metabolites. The computer-generated peak area and the percentage of total radioactivity represented by each peak are shown alongside their retention times (Table 1). We have observed consistently that 3TC triphosphate represents 40% or more of the total intracellular radioactivity of mock-infected PBL after incubation with [^{3}H]3TC for 4 hr, and ~20% or more after 20 hr incubation. Similar results were obtained with HIV-1 infected cells (data not shown).

Intracellular phosphorylation of [^{3}H]3*TC in PHA-stimulated PBL*

[^{3}H]3TC cellular metabolites in HIV-1-infected and mock-infected PBL were analysed by HPLC radiochromatography. 3TC triphosphate levels in PBL from four different donors (A–D) measured up to 20 hr are summarized in Table 2. After 4 hr, 3TC triphosphate levels were similar in HIV-1-infected and mock-infected cells, but varied between donors.

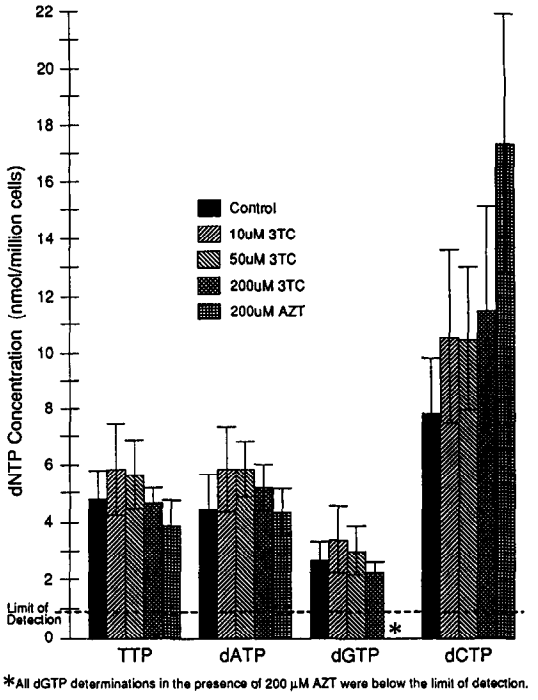
Intracellular decay of [^{3}H]3*TC triphosphate in PHA-stimulated PBL*

In order to determine the rate of decay of 3TC triphosphate, [^{3}H]3TC was incubated with HIV-1- and mock-infected cells for 4 hr to allow detectable

Table 2. 3TC triphosphate levels in HIV-1-infected and mock-infected PBL cultures from four different donors

Donor	Infection status*	Time of exposure (hr) 2	4	20
		(pmol/10^6 cells)		
A	Mock	3.95	6.35	—
	HIV	4.16	6.47	—
B	Mock	12.3	13.4	—
	HIV	12.3	13.8	—
C	Mock	5.69	7.81	—
	HIV	3.74	7.48	—
D	Mock	4.97	4.1	3.7
	HIV	3.13	3.12	3.41

* PHA-stimulated PBL were infected with HIV-1 or mock infected as described in Materials and Methods.



*All dGTP determinations in the presence of 200 μM AZT were below the limit of detection.

Fig. 3. Effects of 3TC and AZT on dNTP levels in U937 cells.

intracellular levels of 5′-phosphorylated metabolites to develop, after which the compound was removed by exhaustive washing, and samples were taken over the following 24 hr. The (+) enantiomer was included for comparison. The rate of decay was determined by plotting dpm values for integrated peak areas of the 5′-triphosphates against sample time where time 0 corresponded to time of removal. The results are summarized in Table 3 as time to a 50% reduction in peak area (dpm) of triphosphate levels from time 0 (half-life).

In mock-infected cells the half-life for 3TC triphosphate was 12 and 15.5 hr in two experiments using different donor PBL. Parallel results for 3TC triphosphate in HIV-1-infected cells showed half-lives of 10.5 and 13.5 hr. In comparison, the triphosphate of the (+) enantiomer demonstrated shorter half-lives in mock-infected cells (3.5 and 3.5 hr) and HIV-1-infected cells (5 and 7 hr).

Effect of 3TC on deoxynucleotide levels in U937 cells

The deoxynucleotide triphosphate levels obtained following treatment with 10, 50 or 200 μM 3TC, or 200 μM AZT are shown in Fig. 3. Each value is the mean ± SEM of five independent determinations. 3TC had no significant effect upon the levels of any of the four deoxynucleotides at any concentration used in this study. In contrast, 200 μM AZT increased dCTP levels and decreased dGTP levels.

The effect of human platelet pyrimidine nucleoside phosphorylase on 3TC

The percentages of the compounds and any products after incubation for various times in platelet-rich suspensions are shown in Table 4. When thymidine and deoxyuridine were incubated in a platelet-rich suspension, the corresponding bases, thymine and uracil, were detected after 30 min. Over 90% of thymidine and deoxyuridine were cleaved to their respective bases after 2 hr. When cytidine was incubated with the platelet-rich suspension, a product peak with retention time and spectrum of uridine

Table 3. Intracellular decay of [^{3}H]3TC 5′-triphosphates in PHA-stimulated PBL

Experiment	[^{3}H]Triphosphate ($T_{1/2}$) Mock-infected (−) Enantiomer (3TC)	Mock-infected (+) Enantiomer	HIV-1-infected (−) Enantiomer (3TC)	HIV-1-infected (+) Enantiomer
1*	15.5	3.5	13.5	5.0
2†	12.0	3.5	10.5	7.0

* Performed using PBL from donor C.
† Performed using PBL from donor B.

Table 4. Percentage of substrate and products after incubation for various times at 37° in a platelet-rich suspension

Compounds	Incubation time (hr)															
	0.5			2			20			45*			93*			
	Nucleoside	Base	Deaminated products	Nucleoside	Base	Deaminated products	Nucleoside	Base	Deaminated products	Nucleoside	Base	Deaminated products	Nucleoside	Base	Deaminated products
Thymidine	40.1	59.1	—	7.8	92.2	—	0	100	—	—	—	—	—	—	—
Deoxyuridine	29.9	70.1	—	3.4	96.3	—	0	100	—	—	—	—	—	—	—
Cytidine	100	—	—	94.1	—	5.9	89	—	11	60.7	5.3	34	24.1	8.2	67.7
(+) Enantiomer	100	—	—	100	—	—	94.8	—	5.2	90.5	—	9.5	84.9	—	14.6
(−) Enantiomer (3TC)	100	—	—	100	—	—	100	—	—	100	—	—	100	—	—

* Separate experiment.

appeared (data not shown) suggesting that cytidine was deaminated to uridine. This peak increased with incubation time and in a separate experiment, after prolonged incubation of cytidine for 45 and 93 hr, another small peak (as well as uridine) was observed. This peak was not identified due to its small size but may be uracil, a degradation product of uridine.

In this study, there were no detectable products of deamination or phosphorolysis when 3TC was incubated with the platelet-rich suspension. However, the (+) enantiomer produced a peak which co-eluted with the deaminated analogue 2′-deoxy-3′-thiauridine, but no base was detected, suggesting that the (+) enantiomer was deaminated but not phosphorolysed. The racemic mixture BCH189 also produced a peak corresponding to 2′-deoxy-3′-thiauridine as a result of deamination of the (+) enantiomer.

DISCUSSION

The cellular phosphorylation of 3TC and the effects of this compound on host cell nucleotide metabolism are described. The intracellular metabolism of [^{3}H]3TC in HIV-1-infected and mock-infected, PHA-stimulated human PBL cultures was analysed *in vitro*. The time-course study of phosphorylation of 3TC has revealed that this compound is phosphorylated to its 5′-triphosphate in both infected and mock-infected cells from different human donors. Despite variation in the extent of phosphorylation between donors, the levels of 5′-triphosphate achieved in the cell (3–13 pmol/10^6 cells) are consistent with the 3TC triphosphate concentration required for activity against purified reverse transcriptase *in vitro* [4]. Moreover, these results show clearly that metabolism of 3TC to its 5′-triphosphate is not dependent on virus infection of cells. We have described elsewhere that both enantiomers have comparable activity against HIV *in vitro* [3]. The 5′-triphosphate derivative of the (+) enantiomer has approximately 5-fold greater activity against purified HIV reverse transcriptase (K_i 8–18 μM dependent on the template/primer system) [4], but as we show here a much shorter half-life in cells than that of 3TC. The intracellular decay of 3TC triphosphate has demonstrated a half-life of 12 and 15.5 hr in mock-infected cells from two donors compared with a half-life of 10.5 and 13.5 hr in HIV-infected cells. The long half-life, without apparent toxicity, of this 3TC product may be relevant to the persistence of antiviral activity in drug-treated patients. In addition, the levels and stability of 3TC triphosphate compare favourably with those achieved by ddC and AZT *in vitro*. ddC triphosphate reaches approximately 10 pmol/10^6 Molt-4 cells after 24 hr with a half-life of 2.6 hr [8]. Low levels of AZT triphosphate have been observed in HIV-1- and mock-infected H9 cells (0.9–1.5 pmol/10^6 cells) with a half-life of approximately 2 hr [9].

Furthermore, the study of the effect of 3TC on cellular deoxynucleotide pools *in situ* has demonstrated no interference at well above therapeutic levels, whereas 200 μM AZT increase dCTP levels and decrease dGTP levels consistent with previous reports [10, 11]. Since 200 μM 3TC is 100–

500-fold higher than its antiviral concentration [3], it is unlikely that the antiviral mechanism involves specific effects upon deoxynucleotide metabolism.

The degradation of nucleoside analogues by phosphorolytic cleavage represents a limitation for their therapeutic potentials. The susceptibility of 3TC to degradation was studied in platelet-rich suspensions which contain thymidine phosphorylase [6]. Whereas thymidine and deoxyuridine were cleaved rapidly to their bases, and the (+) enantiomer was deaminated, 3TC was shown to be resistant to catabolism by human platelets under the same assay conditions. The stability to phosphorolysis is a pivotal property of a compound and it would be of great interest to study the substrate specificities of pyrimidine nucleoside phosphorylase from different tissues, for example the liver or kidney, and to determine whether 3TC is a substrate for the enzyme derived from various tissues.

The activity of an anti-HIV nucleoside *in vivo* is dependent, in part, on the level and stability of its 5′-triphosphate. 3TC fulfils suitably both these criteria and as such is a very promising candidate for anti-HIV chemotherapy.

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