Metabolism, mitochondrial uptake and toxicity of 2',3'-dideoxycytidine

Luigia ROSSI*, Sonja SERAFINI*, Giuditta F. SCHIAVANO†, Anna CASABIANCA*, Giuliana VALLANTI*, Laura CHIARANTINI* and Mauro MAGNANI*¹

*Institute of Biochemistry 'G. Fornaini', University of Urbino, 61029-Urbino, Italy, and †Institute of Hygiene, University of Urbino, 61029-Urbino, Italy

2',3'-Dideoxycytidine (ddCyd) is a prescription anti-retroviral drug that causes mitochondrial toxicity and peripheral neuropathy. ddCyd is actively phosphorylated by cytosolic deoxycytidine kinase and nucleoside (di)phosphate kinase to the 5'-triphosphate derivative. However, 2',3'-dideoxycytidine 5'-diphosphocholine (ddCDP-choline) was also found in human cells incubated with ddCyd. In this paper we show that ddCDP-choline is produced from dideoxyCTP (ddCTP) and phosphocholine by phosphocholine cytidyltransferase. dCTP and CTP appear to activate this synthesis in a concentration-dependent manner. Although ddCTP and ddCDP-choline can both enter the mitochondria, ddCDP-choline uptake is more

efficient than ddCTP uptake. These data suggest that ddCDP-choline is the ddCyd metabolite that is probably responsible for mitochondrial toxicity. The uptake of ddCTP and ddCDP-choline by mitochondria is inhibited by 3.0 mM L-carnitine in the cell-free system investigated; when added to U937 cells grown in the presence of 0.25 μ M ddCyd, 3.0 mM L-carnitine partially abrogated the mitochondrial toxicity of ddCyd.

Key words: L-carnitine, 2',3'-dideoxycytidine 5'-diphosphocholine, mitochondrial carrier, phosphocholine cytidyltransferase.

INTRODUCTION

2',3'-Dideoxycytidine (ddCyd; Zalcitabine), an analogue of the natural nucleoside 2'-deoxycytidine, is effective against HIV-1 *in vitro* [1] and *in vivo* [2,3]. ddCyd exerts its biological effects through its metabolite dideoxyCTP (ddCTP), which inhibits HIV-1 reverse transcriptase. DNA polymerase α , a key enzyme in cellular DNA synthesis, is relatively resistant to the effects of ddCTP, which explains the selective anti-viral effect of ddCyd. However, DNA polymerase β (which is involved in DNA repair) and, in particular, mitochondrial DNA polymerase γ are highly susceptible to inhibition by ddCTP. Studies performed *in vitro* have strongly suggested that the depletion of mitochondrial DNA (mtDNA) could be responsible for the delayed toxicity of ddCyd observed in clinical practice [4]. The major toxic effect of ddCyd that limits the dose that can be used is a painful, reversible, primarily sensory peripheral neuropathy, but ddCyd can also cause granulocytopenia and thrombocytopenia [5]. Interestingly, peripheral neuropathy [6,7] and suppression of haematopoiesis, and the consequent granulocytopenia and thrombocytopenia, have been reported in patients with genetic defects in their mtDNA.

Both cytoplasmic deoxycytidine kinase and mitochondrial deoxypyrimidine kinase can phosphorylate ddCyd to form dideoxyCMP [8]. However, Chen and Cheng [9] demonstrated that the cytoplasmic deoxycytidine kinase is the main enzyme that phosphorylates ddCyd. Thus the phosphorylated ddCyd appears to be transported into mitochondria to exert its inhibitory effect on mtDNA synthesis. ddCTP could enter the mitochondria following conversion into less hydrophilic metabolites, such as 2',3'-dideoxycytidine 5'-diphosphocholine (ddCDP-choline), and/or by carrier-mediated transport of ddCTP. While a mitochondrial carrier capable of transporting dNTPs has been identified [10], nothing is known about the transport of ddCDP-choline. Furthermore, nothing is known about the route of

biosynthesis of ddCDP-choline and, in particular, whether ddCyd must first be converted into ddCTP as a precursor of the ddCDP moiety of the phosphodiester.

In the present paper, the identification, biosynthesis and mitochondrial transport of ddCDP-choline have been investigated. Moreover, since L-carnitine and ddCDP-choline present similar functional chemical groups, we examined the possible effects of L-carnitine on ddCyd-treated U937 cells, on the assumption that L-carnitine could decrease the mitochondrial uptake of ddCDP-choline, thus contributing to prevent ddCyd toxicity.

EXPERIMENTAL

Materials

[³H]ddCTP (6.7 Ci/mmol) and [5,6-³H]ddCyd were obtained from Moravek Biochemicals (La Brea, CA, U.S.A.). Phospho-[methyl-¹⁴C]choline (56.0 mCi/mmol) and [6,6(*n*)-³H]sucrose (16.6 Ci/mmol) were obtained from Amersham. ddCyd was obtained from Fluka (Buchs, Switzerland), and all other reagents from Sigma or Aldrich (Milan, Italy).

Cell culture

U937 monoblastoid cells were grown in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine and antibiotics. U937 cells in exponential-phase growth were seeded into 30 mm-diam. dishes (Costar, Cambridge, MA, U.S.A.) at 5×10^5 cells/ml and grown in an atmosphere of 5% CO₂ in air at 37 °C. ddCyd and/or L-carnitine were added to the culture medium. Half of the culture medium, with or without L-carnitine and ddCyd, was changed every 2 days; at the same time the number of viable cells was determined by the Trypan Blue dye exclusion test using a haemocytometer.

Abbreviations used: ddCDP-choline, 2',3'-dideoxycytidine 5'-diphosphocholine; ddCTP, dideoxyCTP; ddCyd, 2',3'-dideoxycytidine (Zalcitabine); mtDNA, mitochondrial DNA; Rh-123, Rhodamine 123.

¹ To whom correspondence should be addressed: Istituto di Chimica Biologica 'G. Fornaini', Università degli Studi di Urbino, Via Saffi, 2-61029 Urbino, Italy (e-mail magnani@bib.uniurb.it).

Kinetic studies were performed on U937-cell-free supernatants obtained as follows: U937 cells (around 100×10^6) were washed twice in PBS and resuspended in 9 vol. of homogenizing buffer (220 mM mannitol, 70 mM sucrose, 0.5 g/l BSA, 0.2 mM EDTA and 2 mM Hepes, pH 7.4), and homogenized with a Dounce glass homogenizer. The homogenate was centrifuged at 1500 *g* for 10 min to pellet unbroken cells and nuclei. The supernatant was used in some of the studies described below. All procedures were performed at 4 °C.

Phosphocholine cytidyltransferase assay

Phosphocholine cytidyltransferase (EC 2.7.7.15) activity was determined by measuring the rate of incorporation of [*methyl*- ^{14}C]phosphocholine into CDP-choline, dCDP-choline or ddCDP-choline using a charcoal extraction method, essentially as described in [11] with some modifications. Briefly, each reaction mixture contained 1.0 mM [*methyl*- ^{14}C]phosphocholine, 2.0 mM CTP, dCTP or ddCTP, 12 mM magnesium acetate, 50 mM imidazole, 150 mM KCl and 2.0 mM EDTA, pH 7.0, in a total assay volume of 250 μl . The reaction was started by the addition of 50 μl of U937 cell homogenate (4.0 mg of protein/ml), allowed to proceed for 1 h at 37 °C and stopped by the addition of 100 μl of 10% (w/v) trichloroacetic acid/150 mM phosphocholine.

CDP-choline, dCDP-choline or ddCDP-choline was recovered from the reactions by adsorption on to charcoal. The charcoal was collected, washed and eluted using a multiple-sample filtration manifold (Millipore, Bedford, MA, U.S.A.). Cellulose acetate filters (25 mm diam.; pore size 0.45 μm ; Millipore) were used. In detail, the charcoal suspension (1.2 g suspended in 20 ml of water) was stirred continuously in a beaker while 1.0 ml aliquots were removed and added to each reaction tube. After 30 min at room temperature, 7.0 ml of water was added to each tube. After vigorous mixing, the charcoal suspension was collected by centrifugation at room temperature for 5 min at 1500 *g*. The charcoal pellet was suspended in 7.0 ml of water, transferred to the wells of the multiple-sample filtration manifold and collected under reduced pressure. The charcoal pads were washed three times with 7.0 ml of water before the addition of 1.0 ml of elution solvent, consisting of 58 ml of water, 94 ml of ethanol and 5.5 ml of 28% (w/v) NH_4OH . A period of 5 min was allowed for extraction, after which time a vacuum was applied and the extracts were collected in tubes. The extraction procedure was repeated a further three times. Finally, the extracts were placed in scintillation vials, to which 10 ml of scintillation fluid (Emulsifier Scintillator Plus[®]; Packard) was added, and the radioactivity was counted in a Packard Liquid Scintillation Analyzer. Enzyme activity was expressed in nmol of ^{14}C -labelled CDP-choline, dCDP-choline or ddCDP-choline formed/min per mg of protein.

Kinetic properties of phosphocholine cytidyltransferase

To investigate the kinetic properties of phosphocholine cytidyltransferase, 0.25–5.0 mM CTP, dCTP or ddCTP was used as substrate. K_m values were determined from double-reciprocal plots (V^{-1} against S^{-1}). Moreover, phosphocholine cytidyltransferase activity was evaluated using ddCTP at concentrations in the 0.25–1.0 mM range as substrate in the presence of either dCTP or CTP at 0.25–1.0 mM. In this case [^3H]ddCTP was used as the radioactive substrate instead of [*methyl*- ^{14}C]phosphocholine.

As it is not possible to separate [^3H]ddCTP from [^3H]ddCDP-choline by the charcoal extraction method, since both absorb to charcoal, an HPLC method was used. The reaction mixture contained 1.0 mM phosphocholine, 0.25–1.0 mM [^3H]ddCTP

(26614 c.p.m./nmol), 12.5 mM magnesium acetate, 50 mM imidazole, 150 mM KCl and 2.0 mM EDTA, pH 7.0, in a total volume of 250 μl in the absence or presence of dCTP or CTP. The reaction was initiated by the addition of 50 μl of cell homogenate (4.0 mg of protein/ml) and allowed to proceed for 1 h at 37 °C. At 0 and 1 h, 100 μl aliquots were treated for analysis by HPLC. Briefly, samples were extracted with 50 μl of 10% (v/v) HClO_4 , neutralized with K_2CO_3 as reported in [12] and used for HPLC determinations. A 5 μm Res. Elut. 5 C_{18} 90A column (150 mm $\times$ 4.60 mm internal diam.; Varian, Harbor City, CA, U.S.A.) protected with a guard column (Pelliguard LC-18; 20 mm $\times$ 4.60 mm; 40 μm particles) was used. The mobile phase consisted of two eluents: 0.1 M KH_2PO_4 and 5 mM tetrabutylammonium hydroxide solution adjusted to pH 4.95 with 1.0 M KH_2PO_4 (buffer A), and buffer A containing 30% (v/v) methanol (buffer B). All buffer solutions, as well as standards and sample solutions, were filtered through a 0.22 μm -pore-size membrane filter (Millipore). The elution conditions were: 5 min at 100% buffer A, up to 100% buffer B over 25 min, held at 100% buffer B for 5 min, after which the gradient was returned to 100% buffer A over 2 min and the initial conditions restored in 3 min. The analyses were performed at room temperature; the flow rate was 1.0 ml/min and the detection wavelength was 272 nm. The retention times under the conditions used were 7.5 min for ddCDP-choline, 13 min for ddCyd and 16.5 min for ddCTP. Fractions (0.5 ml) were collected at the exit of the wavelength detector with an LKB fraction collector, and radioactivity was counted in a Packard 1500 liquid scintillation counter. The amount of radioactivity collected at the retention time of ddCDP-choline (7.5 min) was taken as the amount of ddCDP-choline present in the reaction mixture.

Preparation of mitochondria

Mitochondria from rabbit liver were prepared using a modified version of the procedure described in [13]. Briefly, approx. 10 g of liver was chopped with scissors and homogenized in a Potter–Elvehjem homogenizer in 10 vol. of homogenizing buffer with a Teflon pestle (clearance 0.1 mm). The homogenate was centrifuged for 10 min at 1500 *g*; the supernatant was then carefully decanted and centrifuged at 6500 *g* for 15 min to obtain the crude mitochondrial pellet. This pellet was resuspended in 40 ml of homogenizing buffer, homogenized with a Dounce glass homogenizer and centrifuged as above. This procedure was repeated twice. The final pellet was resuspended in 5 ml of homogenizing buffer and used in the studies described below. The activities of lactate dehydrogenase (EC 1.1.1.27) and glutamate dehydrogenase (EC 1.4.1.3.) in the rabbit liver mitochondria suspension were determined as described in [14] and [15] respectively, and found to be 0.11 ± 0.035 unit/mg of protein for lactate dehydrogenase and 7.95 ± 2.54 units/mg of protein for glutamate dehydrogenase. These values confirm that the mitochondrial fraction was virtually free of cytosol. The protein content was determined using Bradford's protein assay reagent (Bio-Rad), with the homogenizing buffer as blank.

ddCTP uptake by intact mitochondria

Rabbit liver mitochondria (6.2 mg of protein/ml of homogenate) were incubated with 0.1, 1.0 or 2.0 μM [^3H]ddCTP (19233 c.p.m./pmol) in homogenizing buffer in the absence or presence of dCTP (0.5–2.0 μM) or CTP (0.5–2.0 μM), for 10 min at room temperature in a final volume of 350 μl . At 0, 5 and 10 min, a 100 μl aliquot was layered on to 100 μl of 10% (w/v) Ficoll solution and centrifuged at 14000 *g* for 4 min. After centrifugation and subsequent stratification, the pellets were

separated by freezing the tube and cutting off the tip at the bottom part of the Ficoll layer nearest the pellet layer. The pellets were removed with 200 μ l of double-distilled water, and 200 μ l of Soluene-350 (Packard) was added. After 1 h of incubation at room temperature, 4.5 ml of scintillation fluid (Packard) was added to each sample; the radioactivity was counted in a Packard 1500 liquid scintillation counter and the amount of [3 H]ddCTP taken up by the mitochondria was evaluated. In a parallel experiment, [3 H]sucrose (65000 c.p.m.) was added to mitochondria instead of [3 H]ddCTP, in order to evaluate the amount of radioactivity non-specifically incorporated into the pellet. This value (less than 1% of the total) was subtracted from the [3 H]ddCTP value. When the uptake of ddCTP and ddCDP-choline was studied in parallel experiments, 5–20 mM ddCTP was added to rabbit liver mitochondria.

ddCDP-choline uptake by intact mitochondria

Rabbit liver mitochondria (2.0 mg of protein/ml of homogenate) were incubated in homogenizing buffer with [3 H]ddCDP-choline (21 734 c.p.m./nmol) at concentrations ranging from 5 to 20 mM for 10 min at room temperature in a final volume of 110 μ l for each time point (0, 2, 5 and 10 min). At the end of each incubation period, a 100 μ l aliquot was layered on to Ficoll and the same procedure as described for [3 H]ddCTP uptake was followed. In a further experiment, the uptake of [3 H]ddCDP-choline by rabbit mitochondria was evaluated in the presence of 3.0 mM L-carnitine.

[3 H]ddCDP-choline was synthesized starting from 1.0 mM ddCTP in the presence of [3 H]ddCTP (21 734 c.p.m./nmol) and 1.0 mM phosphocholine in a final volume of 500 μ l. The reaction was initiated by the addition of 200 μ l of U937 cell homogenate (2.44 mg of protein/ml) and allowed to proceed for 3 h at 37 °C, after which time the sample was treated for HPLC as described above. Fractions were collected at the exit of the wavelength detector from 6 to 9 min, concentrated with a SpeedVac Concentrator (Savant Instruments) and resuspended in double-distilled water. Purification of [3 H]ddCDP-choline from salts (see buffers A and B) was performed by absorption to charcoal. After the elution step, the sample was concentrated and resuspended in double-distilled water to a final volume of 500 μ l, containing 0.2 nmol of ddCDP-choline/ μ l. The final specific activity was 21 734 c.p.m./nmol, as for the precursor [3 H]ddCTP.

mtDNA content

DNA was isolated from U937 cells using a lysis buffer (100 μ l of lysis buffer per 250 000 cells) containing 8 M urea, 0.3 M NaCl and 10 mM Tris/HCl, pH 7.5, for 20 min at 37 °C. Extraction was with phenol/chloroform/3-methylbutan-1-ol (25:24:1, by vol.) and then with chloroform/3-methylbutan-1-ol (24:1, v/v). The DNAs were precipitated with ethanol and stored at –20 °C until use.

For each sample, 1 μ g of total cellular DNA was digested with the restriction enzyme *Bam*HI, analysed on a 0.8% (w/v) agarose gel and transferred to a nylon membrane under the conditions recommended by the manufacturer (Amersham). To detect the mtDNA on the nylon membrane, a 1237 bp mtDNA fragment labelled with 32 P was used as a probe. The mtDNA probe (kindly provided by Dr. M. N. Gadaleta, University of Bari, Italy) spans nucleotides 6204–7441 of complete human mtDNA [16]. The DNA probe was labelled using the random primer DNA-labelling kit from Bio-Rad. DNA hybridization was carried out as described in the instructions for DNA hybridization supplied by Amersham. In this experiment, increasing amounts of a plasmid pUC18 containing a 1237 bp mtDNA fragment were used as a

standard (pUC18-1237m). The intensities of the radiolabelled bands were quantified in a GS-250 molecular imager (Bio-Rad).

Cytofluorimetric analysis of U937 cell lines stained with Rhodamine 123 (Rh-123)

Cell samples (0.5×10^6 cells/ml) were collected during the exponential phase of growth and incubated in complete medium for 30 min at 37 °C in the dark with 10 μ g/ml Rh-123. After incubation, the cells were washed twice in Sheath Fluid (Becton Dickinson, San Jose, CA, U.S.A.), resuspended in 400 μ l of Sheath Fluid and analysed. Cytofluorimetric analysis was performed with a FACScan instrument (Becton Dickinson). A minimum of 10 000 events per sample were analysed. Data were acquired in list mode and analysed with Lysys II software (Becton Dickinson).

RESULTS

ddCyd metabolism in U937 cell lysates

In human cells, ddCyd is rapidly converted into ddCTP and dideoxyliponucleotides (mainly ddCDP-choline) [17–19]. Furthermore, cells exposed to ddCyd undergo a time-dependent depletion of mtDNA [18]. The biochemical basis of this event is poorly understood. ddCyd is initially phosphorylated to dideoxy-CMP by a cytosolic deoxycytidine kinase. In fact, deoxycytidine kinase mutant cell lines do not accumulate phosphorylated ddCyd and are resistant to its mitochondrial toxicity [9]. Furthermore, in quantitative terms, the most important forms of ddCyd in human cells are the deoxyliponucleotide derivatives ddCDP-choline and ddCDP-ethanolamine [19]. With this in mind, we investigated ddCyd metabolism in U937 cell-free supernatants.

As a starting point for assessing the origin of the ddCDP-choline in U937 cell homogenates, we assumed a direct biosynthesis analogous to the synthesis of CDP-choline via CTP:phosphocholine cytidyltransferase. To this end, phosphocholine cytidyltransferase activity was assessed using a concentration range 0–5.0 mM (0, 0.25, 0.5, 1.0, 2.0 and 5.0 mM) for CTP, dCTP and ddCTP as substrates at a fixed phosphocholine concentration (1 mM), as described in the Experimental section.

The results shown in Table 1 were obtained using Lineweaver–Burk double-reciprocal plots of the amounts of CDP-choline, dCDP-choline and ddCDP-choline produced. Although similar K_m values were found for the three different substrates tested, the efficiency of phosphocholine cytidyltransferase for CTP and dCTP as substrates was three times greater than for ddCTP. However, the U937 cell homogenate was able to produce ddCDP-choline starting from ddCTP with a V_{max} of 0.38 ± 0.02 nmol/min per mg of protein.

Table 1 Kinetic properties of CTP:phosphocholine cytidyltransferase

All determinations were performed using U937 cell-free supernatants at 37 °C. Values are means $\pm$ S.D. of three experiments.

Substrate	K_m (mM)	V_{max} (nmol/min per mg of protein)	Efficiency for substrate (V_{max}/K_m)
CTP	0.87 ± 0.05	1.15 ± 0.08	1.3
dCTP	1.07 ± 0.08	1.11 ± 0.10	1.03
ddCTP	0.94 ± 0.05	0.38 ± 0.02	0.4

Figure 1 ddCDP-choline formation in the absence and in the presence of dCTP or CTP

ddCDP-choline formation was evaluated using different amounts of [^3H]ddCTP in the presence of various dCTP (**A**) or CTP (**B**) concentrations. Determinations were performed on U937 cell homogenates after a 1 h incubation at 37 °C. Values are expressed in nmol/h per mg of protein, and are the means of three experiments which agreed within 5.0%.

In an attempt to investigate ddCDP-choline formation under conditions as close as possible to those found in intact cells, we studied the effects of CTP and dCTP on the rate of ddCDP-choline synthesis. Since the charcoal method is unable to separate [^3H]ddCTP from [^3H]ddCDP-choline, we used an HPLC assay (see the Experimental section). The results obtained show that the formation of ddCDP-choline was stimulated especially at low dCTP (or CTP) concentrations (Figure 1), while it remained unchanged at high dCTP concentrations, suggesting that dCTP (or CTP) can act as an activator of phosphocholine cytidyl-transferase activity.

Taken together, our results show that U937 cells are able to produce ddCDP-choline starting from ddCTP. This means that, following ddCyd treatment, cells produce not only ddCTP, which is active against HIV reverse transcriptase, but also the phosphodiester ddCDP-choline, the biological significance of which is unknown.

Uptake of ddCTP and ddCDP-choline by intact mitochondria

We reasoned that the phosphodiester metabolite ddCDP-choline, which is less hydrophilic than ddCTP, might enter the mitochondria and act as a reservoir form of ddCTP that is responsible for the inhibition of the mtDNA polymerase γ , explaining the delayed toxicity of ddCyd. We first examined the uptake of ddCTP by rabbit liver mitochondria. ddCTP was able to cross the mitochondrial membrane in a concentration-dependent manner. Substrate competition experiments showed that ddCTP transport could be reduced by both dCTP and CTP. K_m and V_{max} values are shown in Table 2. Our results confirmed those obtained by other authors [10], indicating that ddCTP is able to enter the mitochondrion.

The mitochondrial uptake of ddCTP and ddCDP-choline was then compared. To this end, ddCDP-choline was produced starting from ddCTP and phosphocholine in the presence of a U937 cell homogenate, then separated and purified as described in the Experimental section. Rabbit liver mitochondria were incubated with different concentrations of ddCDP-choline

Table 2 Kinetic properties of the ddCTP mitochondrial carrier

K_m values were obtained from a double-reciprocal plot (V^{-1} against S^{-1}), while K_i values were obtained from the slope replotted against the dCTP or CTP concentration. Values are means $\pm$ S.D. of three determinations.

Parameter	Value
K_m (μM)	1.82 ± 0.20
V_{max} (nmol/min per g of protein)	2.50 ± 0.01
K_i (dCTP) (μM)	0.82 ± 0.05
K_i (CTP) (μM)	0.45 ± 0.03

(5–20 μM). Unfortunately, the specific radioactivity of this compound was only 21734 c.p.m./nmol, and concentrations lower than 5 μM could not be tested without significant errors. The concentration of ddCDP-choline inside the mitochondria was evaluated at different times of incubation and found to be a linear function of time in the interval tested (0–10 min). The results obtained showed that the uptake of ddCDP-choline in mitochondria was saturated at all concentrations tested, with a V_{max} of 10.9 ± 0.24 nmol/min per g of protein. When the uptake of ddCTP and ddCDP-choline was studied in parallel experiments, different affinities of the mitochondrial carrier system for the two compounds were observed: ddCDP-choline uptake was saturated at 5 μM , while ddCTP uptake was not saturated (at least at 20.0 μM). Furthermore, the V_{max} for ddCTP uptake was four times lower than that for ddCDP-choline uptake, suggesting that ddCDP-choline is probably the preferred ddCyd metabolite for entry into mitochondria.

Effect of L-carnitine in ddCyd-treated U937 cells

Since L-carnitine and ddCDP-choline present a similar trimethyl C-terminal end, we repeated the above experiments in the

Figure 2 Determination of mtDNA content in U937 cells

Total cellular DNA was extracted from portions of 1×10^6 cells exposed to ddCyd and/or to L-carnitine for 10 days. Each sample of DNA (0.5 μg; corresponding to approx. 0.25×10^5 cells) was analysed as described in the Experimental section. As a standard curve, increasing copy number of a linearized pUC18-1237m is shown. The percentage mtDNA content is with respect to untreated U937 cells (100%), and values are means $\pm$ S.D. of three experiments.

presence of L-carnitine in order to evaluate a potential competitive effect on ddCDP-choline uptake. We found that 3 mM L-carnitine was able to decrease the uptake of ddCDP-choline and ddCTP by isolated mitochondria by about 90% (results not shown). As a consequence, we decided to evaluate a possible protective effect of carnitine by investigating the mtDNA content and the percentage of cells with functioning mitochondria following treatment with ddCyd.

For this purpose, U937 cells were grown for 10 days in the presence of 0.25 or 1.0 μM ddCyd, in either the absence or the presence of 1.0 or 3.0 mM L-carnitine. After 10 days of treatment, the mtDNA content and percentage of fluorescent Rh-123-positive cells were evaluated. The results obtained (Figure 2) confirmed the mitochondrial cytotoxicity caused by ddCyd treatment, as previously reported [18], while an increase in mtDNA content was observed upon L-carnitine treatment. In particular, the amount of mtDNA obtained doubled when U937 cells were grown in the presence of 0.25 μM ddCyd and 3.0 mM L-carnitine. Moreover, upon ddCyd treatment, a slight decrease (15%) in the percentage of cells containing functioning mitochondria (Rh-123-positive) was observed. On the addition of L-carnitine (both 1.0 and 3.0 mM), the percentage of fluorescent Rh-123-positive cells was restored to 83%, the same value observed in the untreated control U937 cells (results not shown).

DISCUSSION

The observation by Kennedy and Weiss [20] that CTP is required for the enzymic formation of phospholipids led to the discovery of the two riboliponucleotides, CDP-choline and CDP-ethanolamine, and the elucidation of their synthesis from CTP plus phosphocholine (or phosphoethanolamine) by specific cytidylyl-

transferase activities [21]. Subsequently it was found that these enzymes are able to use dCTP instead of CTP to form the corresponding deoxyliponucleotides, dCDP-choline and dCDP-ethanolamine [22]. In the present paper we have shown that phosphocholine cytidylyltransferase can also produce ddCDP-choline starting from ddCTP, the active form of the anti-retroviral drug ddCyd. We have also shown that phosphocholine cytidylyltransferase uses CTP and dCTP with an efficiency that is about three times higher than that with ddCTP. Furthermore, in the presence of CTP or dCTP, ddCDP-choline production increases, suggesting that CTP or dCTP acts as an activator of phosphocholine cytidylyltransferase activity, at least at low concentrations. However, at a concentration of 1.0 mM, dCTP appeared to be a better inhibitor than CTP when 0.5 mM ddCTP was used (see Figure 1).

In addition, we have demonstrated that both ddCTP (confirming reports by other authors [10]) and ddCDP-choline are transported into mitochondria. While the presence of ddCTP inside mitochondria could be closely linked to the depletion of mtDNA and consequently to the delayed ddCyd toxicity, nothing is known about the biological role of ddCDP-choline. However, we previously demonstrated [18] that, under steady-state conditions, ddCyd accumulates preferentially in the cell in the form of ddCDP-choline rather than ddCTP, and we have now shown that ddCDP-choline is transported into the mitochondrion more efficiently than is ddCTP. In view of the reversibility of the phosphocholine cytidylyltransferase reaction [21,23], we suggest that the ddCDP-choline inside mitochondria could act as a reservoir form of ddCTP, thus contributing to mtDNA depletion. Unfortunately, this process is very slow (6 h of incubation in the presence of 0.1 μM ddCyd is required to obtain a 55% decrease in U937 cell mtDNA content), and cell-free experiments cannot be performed reproducibly over such long periods. On the other hand, experiments performed using intact cells did not allow us to differentiate between the two potential precursors.

ddCDP-choline may also interfere with the synthesis of phosphatidylcholine, causing a perturbation of the lipid neuronal membrane bilayer and contributing, at least in part, to the sensory peripheral neuropathy observed after ddCyd treatment, as previously suggested [24].

Two separate dCTP pools, one (labelled from cytidine; named dCTP₂) required for the synthesis of DNA and the other (labelled from deoxycytidine; named dCTP₁) required for the synthesis of deoxyliponucleotides, have been identified in the same cell [25–27]. In previous studies [28] we demonstrated that ddCyd concentrations higher than 1.0 μM (where 0.1 μM is the therapeutic concentration) cause inhibition of cell growth due to incorporation of ddCTP into cellular DNA. Thus high ddCTP concentrations can compete with the dCTP₂ pool for incorporation into nuclear DNA. In contrast, the existence in the cell of two separate pools of dCTP is of limited interest in understanding ddCTP and ddCDP-choline metabolism at low ddCyd concentrations. In fact, neither CTP nor dCTP was a significant inhibitor of phosphocholine cytidylyltransferase when ddCTP was used in the 0.1–1.0 μM concentration range.

Since ddCDP-choline is the preferred ddCyd metabolite for transport into the mitochondria, we reasoned that the identification of a compound able to counteract this transport might decrease the mitochondrial toxicity of ddCyd. As L-carnitine and ddCDP-choline present a similar trimethyl C-terminal end, and since a mitochondrial carrier for carnitine is well known [29], L-carnitine could have a competitive effect on ddCDP-choline uptake. Whereas in a cell-free system 3.0 mM L-carnitine almost completely inhibited the mitochondrial uptake of both ddCTP and ddCDP-choline, when added to intact U937 cells it was not

able to completely prevent the decrease in mtDNA caused by ddCyd. However, it is noteworthy that a 2-fold greater amount of mtDNA was maintained in cells grown in the presence of 0.25 μ M ddCyd when 3.0 mM L-carnitine was added. These data suggest that L-carnitine may counteract the mitochondrial uptake of ddCDP-choline, but further experiments will be necessary before using it for treatments in humans.

In conclusion, our results show that the phosphodiester ddCDP-choline is produced starting from ddCTP, i.e. after activation of the anti-retroviral drug ddCyd. Both ddCTP and, more efficiently, ddCDP-choline are able to enter the mitochondria and can directly (ddCTP) or indirectly (ddCDP-choline) inhibit DNA polymerase γ and cause a decrease in the mtDNA content, thus playing a role in the delayed toxicity of ddCyd. Simultaneously, ddCDP-choline may also interfere with lipid membrane biosynthesis, contributing to the neurotoxicity observed in AIDS patients after ddCyd treatment.

This work was supported by the C.N.R. Target Project on Biotechnology and Ministero della Sanità, Istituto Superiore di Sanità (Programma Nazionale di Ricerca sull'AIDS, 1998), Rome, Italy.

REFERENCES

- Mitsuya, H. and Broder, S. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 1911–1915
- Merigan, T. C., Skowron, G., Bozzette, S. A., Richman, D., Uttamchandani, R., Fischl, M., Schooley, R., Hirsch, M., Soo, W., Pettinelli, C. et al. (1989) *Ann. Intern. Med.* **110**, 189–194
- Yarchoan, R. and Broder, S. (1987) *N. Engl. J. Med.* **316**, 557–564
- Chen, C. H. and Cheng, Y. C. (1989) *J. Biol. Chem.* **264**, 11934–11937
- Merigan, T. C. and Skowron, G. (1990) *Am. J. Med.* **88**, 11S–15S
- Peyronnard, J. M., Charron, L., Bellavance, A. and Marchand, L. (1980) *Ann. Neurol.* **7**, 262–268
- Pezeskpour, G., Krarup, C., Buchthal, F., Di Mauro, S., Bresolin, N. and McBurney, J. (1987) *J. Neurol. Sci.* **77**, 285–304
- Starnes, M. C. and Cheng, Y. C. (1987) *J. Biol. Chem.* **262**, 988–991
- Chen, C. H. and Cheng, Y. C. (1992) *J. Biol. Chem.* **267**, 2856–2859
- Bridges, E. G., Jiang, Z. L. and Cheng, Y. C. (1997) *Proc. Am. Assoc. Cancer Res.* **38**, 414
- Weinhold, P. A. and Feldman, D. A. (1992) *Methods Enzymol.* **209**, 248–251
- Brandi, G., Rossi, L., Schiavano, G. F., Salvaggio, L. and Magnani, M. (1991) *Chem. Biol. Interact.* **79**, 53–64
- Lai, J. C. K. and Clark, J. B. (1979) *Methods Enzymol.* **55**, 51–60
- Beutler, E. (1975) in *Red Cell Metabolism: A Manual of Biochemical Methods*, 2nd edn., pp. 65–66, Grune and Stratton, New York
- Smidt, E. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. K., ed.), pp. 650–656, Academic Press, New York
- Anderson, S., Bankier, A. T., Barrell, B. G., de Bruijn, M. H., Coulson, A. R., Drouin, J., Eperon, I. C., Nierlich, D. P., Roe, B. A., Sanger, F. et al. (1981) *Nature (London)* **290**, 457–465
- Cooney, D. A., Dalal, M., Mitsuya, H., McMahon, J. B., Nadkarni, M., Balzarini, J., Broder, S. and Johns, D. G. (1986) *Biochem. Pharmacol.* **35**, 2065–2068
- Magnani, M., Brandi, G., Casabianca, A., Fraternali, A., Schiavano, G. F., Rossi, L. and Chiarantini, L. (1995) *Biochem. J.* **312**, 115–123
- Hao, Z., Stowe, E. E., Ahluwalia, G., Baker, D. C., Hebbler, A. K., Chisena, C., Musser, S. M., Kelley, J. A., Perno, C. F., Johns, D. G. and Cooney, D. A. (1993) *Drug Metab. Dispos.* **21**, 738–744
- Kennedy, E. P. and Weiss, S. B. (1955) *J. Am. Chem. Soc.* **77**, 250–251
- Kennedy, E. P. and Weiss, S. B. (1956) *J. Biol. Chem.* **222**, 193–214
- Kennedy, E. P., Borkenhagen, L. F. and Smith, S. W. (1959) *J. Biol. Chem.* **234**, 1998–2000
- Feldman, D. A., Kovac, C. R., Dranginis, P. L. and Weinhold, P. A. (1978) *J. Biol. Chem.* **253**, 4980–4986
- De Groote, K., Naesens, L., Balzarini, J., Baes, M. I. and De Clercq, P. E. (1997) *Biochem. Pharmacol.* **54**, 713–719
- Spyrou, G. and Reichard, P. (1987) *J. Biol. Chem.* **262**, 16425–16432
- Sasvari-Szekely, M., Spasokukotskaja, T. and Staub, M. (1993) *Biochem. Biophys. Res. Commun.* **194**, 966–972
- Spasokukotskaja, T., Sasvari-Szekely, M. and Staub, M. (1992) *FEBS Lett.* **297**, 151–154
- Brandi, G., Rossi, L., Schiavano, G. F., Salvaggio, L., Albano, A. and Magnani, M. (1991) *Chem. Biol. Interact.* **79**, 53–64
- Palmieri, F., Indiveri, C., Bisaccia, F. and Iacobazzi, V. (1995) *Methods Enzymol.* **260**, 349–369

Received 12 July 1999/9 September 1999; accepted 11 October 1999