2′,3′-Dideoxycytidine metabolism in a new drug-resistant cell line

Mauro MAGNANI,*‡ Giorgio BRANDI,† Anna CASABIANCA,* Alessandra FRATERNALE,* Giuditta F. SCHIAVANO,† Luigia ROSSI* and Laura CHIARANTINI*

*Istituto di Chimica Biologica 'G. Fornaini', Università degli Studi di Urbino, 61029 Urbino, Italy, and †Istituto di Igiene, University of Urbino, Italy

2′,3′-Dideoxycytidine (ddC) is a nucleoside analogue that inhibits human immunodeficiency virus type 1 (HIV-1) replication *in vitro* and is currently used in the therapy of acquired immune deficiency syndrome (AIDS). This compound exerts a delayed cytotoxicity due to inhibition of mitochondrial DNA (mDNA) synthesis. Long-term exposure of U937 human monoblastoid cells to ddC resulted in a time- and concentration-dependent decrease in mDNA content and Rhodamine 123 fluorescence. However, after 2 months on 0.1 μM ddC, a drug-resistant cell line (U937-R) with 66% of the normal amount of mDNA was isolated. ddC transport in U937 and U937-R cell lines was similar. In contrast, U937-R accumulated ddC phosphorylated derivatives at a much lower rate and to a reduced concentration into acid-soluble material. The rate of 2′,3′-dideoxycytidine 5′-triphosphate (ddCTP) formation in U937-R cells was almost one-third of that measured in normal cells, although the rate of ddCTP catabolism was similar in both cell lines. Dideoxylipo-nucleotide (ddCDP-choline and ddCDP-ethanolamine) formation was also much slower (between one-half and one-third as fast) in U937-R than in control cells, although catabolism occurred at similar rates. ddC was phosphorylated by a cytoplasmic deoxycytidine kinase in both cell lines. This enzyme showed K_m values for ddC of 80 ± 7 and 140 ± 9 μM in U937 and U937-R cells respectively. Furthermore, V_{max} was 12 ± 1.1 and 7.8 ± 0.5 pmol/min per mg of protein in U937 and U937-R. Thus resistance to ddC toxicity may be due to cells' decreased ability to accumulate intracellular ddC anabolites, which may depend on cytoplasmic deoxycytidine kinase.

INTRODUCTION

A number of nucleoside analogues have been used successfully both as anticancer and antiviral agents for many years. Unfortunately, these drugs have toxic side effects that concern essentially proliferating tissues (i.e. bone marrow and gastrointestinal tract) when the nucleoside analogues are given over short periods, or damage to mitochondrial function when the drugs must be given over long periods of time [1]. 2′,3′-Dideoxycytidine (ddC) is one of the most potent human immunodeficiency virus (HIV) inhibitors *in vitro* [2] and has shown beneficial effects in patients with acquired immune deficiency syndrome (AIDS) [3]. After several weeks on ddC treatment, some patients develop a peripheral neuropathy [4]. Studies *in vitro* have strongly suggested that the depletion of mitochondrial DNA (mDNA) could be responsible for the delayed toxicity of ddC observed clinically [5,6]. This depletion of mDNA may be due to inhibition of DNA polymerase γ by 2′,3′-dideoxycytidine 5′-triphosphate (ddCTP) [7]. The cytoplasmic ddC kinase seems to be the enzyme responsible for the initial phosphorylation of ddC [6], and in turn for ddC toxicity.

Two additional problems are associated with the use of reverse transcriptase (RT) inhibitors and are likely to contribute to the failure of existing therapies in completely blocking clinical progression of AIDS. The first reason why individuals progress to disease while on antiretrovirals can be found in the frequent changes of HIV genomic sequences in the same patient due to the high error rate of RT; the second reason is probably the failure to maintain adequate drug levels in any potential site of viral replication over long periods [8]. Although RT mutations that confer resistance to the nucleoside analogues have been investigated [9,10], drug metabolism in cells and tissues maintained at adequate drug concentrations for extended periods has been poorly studied. In this paper we have investigated ddC toxicity in U937 human monoblastoid cells exposed to the drug for several weeks. Furthermore, we have shown that it is possible to isolate ddC-resistant cells (U937-R) with high frequency and that the resistance to ddC toxicity is associated with a reduced cell ability to accumulate intracellular ddC anabolites.

EXPERIMENTAL PROCEDURES

Materials

2′,3′-[5,6-^{3}H]Dideoxycytidine (6 Ci/mmol) and 2′-[5-^{3}H]deoxycytidine (20 Ci/mmol) were obtained from Moravek Biochemicals (La Brea, CA, U.S.A.). [U-^{14}C]sucrose (350 mCi/mmol) was obtained from Amersham. ddC was obtained from Fluka (Buchs, Switzerland) and the other reagents from Sigma or Aldrich (Milan, Italy).

Cell culture

U937 monoblastoid cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine and antibiotics.

The ddC-resistant cell line U937-R was isolated after exposure of U937 cells to ddC for at least one month. Briefly, log-phase cells (5×10^5 cells per ml) were treated with 0.1 μM ddC in 25 mm-diam. dishes and grown in an atmosphere of 5% (v/v) CO_2 in air at 37 °C. Half of the culture medium and ddC were changed every 2 days; at the same time the number of viable cells was determined with the Trypan Blue dye exclusion test by using a haemocytometer.

To test whether the ability of U937-R to grow in the presence of ddC was maintained even in the absence of the drug, U937 cells were first grown in the presence of 0.1 μM ddC, then in the absence of drug for at least 15 days and finally grown again in the presence of 0.1 μM ddC.

Abbreviations used: AIDS, acquired immune deficiency syndrome; dC, 2′-deoxycytidine; ddC, 2′,3′-dideoxycytidine; ddCTP, 2′,3′-dideoxycytidine 5′-triphosphate; HIV-1, human immunodeficiency virus type 1; mDNA, mitochondrial DNA; Rh-123, Rhodamine 123; RT, reverse transcriptase.

‡ To whom correspondence should be addressed.

Measurement of mDNA

DNA was isolated from U937 and U937-R cells by a lysis buffer (100 μl of lysis buffer per 250000 cells) containing 8 M urea, 0.3 M NaCl, 10 mM Tris/HCl, pH 7.5, for 60 min at 37 °C. Extraction was with phenol/chloroform/isoamyl alcohol (25:24:1, by vol.) and then with chloroform/isoamyl alcohol (24:1, v/v). The DNA was precipitated with 3 volumes of ethyl alcohol absolute and the precipitate was then dissolved in water.

Total cellular DNA was digested with the restriction enzyme *Bam*HI, analysed on a 0.8% agarose gel and transferred to a nylon membrane under the conditions recommended by the manufacturer (Amersham). To detect the mitochondrial DNA on the nylon membrane, a 1544 bp mitochondrial DNA fragment labelled with ^{32}P was used as a probe. The mitochondrial DNA probe (kindly provided by Dr. Gadaleta, University of Bari, Italy) spans from nucleotide 2578 to 4122 in the complete human mitochondrial DNA [11]. Labelling of the DNA probe was by the random primer DNA-labelling kit from Bio-Rad. DNA hybridization was carried out as described in the brochure for DNA hybridization supplied by Amersham. The relative amount of mitochondrial DNA in each sample of treated or resistant cells compared with the control cells was determined by scanning the autoradiograph with an LKB Ultrascan XL laser scan densitometer.

Cytofluorimetric analysis of U937 cell lines stained with Rhodamine 123

Cell samples (0.5×10^6 cells per 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 μg/ml of Rhodamine 123 (Rh-123). After incubation the cells were washed twice in Sheath Fluid (Becton Dickinson, San José, CA, U.S.A.), resuspended in 400 μl of Sheath Fluid and analysed. Cytofluorimetric analysis was performed with a FACScan (Becton Dickinson). A minimum of 10000 events per sample were analysed. Data were acquired in list mode and analysed with Lysys II software (Becton Dickinson).

ddC metabolism

[5,6-^{3}H]Dideoxycytidine incorporation into phosphorylated metabolites was measured by incubation of U937 and U937-R cells at a density of 0.8×10^6 to 1.0×10^6 cells per ml in six-well plates in the presence of different ddC concentrations and for different incubation times (1–48 h). At each time and for each concentration tested, duplicate cell samples were harvested by centrifugation after the addition of an excess of ice-cold PBS, after which the radioactive culture media was decanted and the cell pellets were extracted with 250 μl of 5% (v/v) $HClO_4$. The $HClO_4$ extract was neutralized with K_2CO_3 as reported in [12], for analysis by HPLC to determinate metabolic products of ddC. Total phosphorylated metabolites of ddC were measured by adsorption of neutralized $HClO_4$ extracts on DE-81 filter discs that were washed and counted as in [13]. ddC incorporated into DNA was determined as in [12].

HPLC chromatography was done on a 3 μm Supelcosil LC-18T column (15 cm × 4.6 mm int. diam.) protected by a guard column (Pelliguard LC-18, 20 mm × 4.6 mm int. diam., 40 μm particles). The mobile phase consisted of two eluants: 0.1 M KH_2PO_4, 5 mM tetrabutylammonium hydroxide solution adjusted to pH 4.95 by 1 M KH_2PO_4 (buffer A) and buffer A containing 30% (v/v) methanol (buffer B). The elution conditions were: 5 min of 100% buffer A, up to 100% buffer B over 25 min and hold for 5 min. The gradient was returned to 100% buffer A over 2 min and the initial conditions restored over 4 min. The flow rate was 1.0 ml/min and the detection wavelength was 272 nm. Analyses were performed at room temperature and quantitative measurements were obtained by injection of a standard of known concentration. The molar absorption value used for standard calibration of ddC, ddCMP, ddCDP and ddCTP at 272 nm was 9.1. The retention times for ddC, ddCMP, ddCDP and ddCTP were 9.5, 10.5, 11.5 and 17.0 min respectively. Fractions (0.3 ml) were collected at the exit of the wavelength detector with an LKB fraction collector and counted in a Packard 1500 liquid scintillation counter.

ddC transport measurements

Assays of ddC influx into U937 and U937-R cells were performed at 37 °C in 1.5 ml polypropylene microcentrifuge tubes, using an 'oil-stop' method [14]. Uptake intervals were initiated by the rapid addition of 350 μl of cell suspension (5×10^5 cells) to an equal volume of ^{3}H-permeant solution layered over 600 μl of 1-bromododecane in 1.5 ml microcentrifuge tubes. Uptake was ended by centrifugal pelleting of the cells at 20 s intervals. The ^{3}H content of cell pellets was measured as in [14]. The amount of extracellular medium in the cell pellet was determined with [U-^{14}C]sucrose as previously reported [15]. Initial velocities were determined during the linear phase of influx, which was within 2 min.

ddC kinase assay

The ddC kinase reaction mixture contained, in a final volume of 120 μl, 6 mM ATP, 6 mM $MgCl_2$, 7 mM KF, 0.2% (w/v) BSA, 0.1 M Tris/HCl, pH 7.5, 2.4×10^6 c.p.m. [^{3}H]2′-deoxycytidine (dC) and [^{3}H]ddC in the range 100–500 μM. When dC was used as a substrate it was at 10–100 μM containing 2.1×10^6 c.p.m. [^{3}H]dC. Incubation was for 1 h at 37 °C. The assay was linear during this time. An aliquot of the reaction mixture (100 μl) was spotted on to a DE-81 disc, washed 3 times with 1 mM ammonium formate, once with water and once with ethanol as in Ref. [16], and counted in a β-counter (Packard Model 1500). K_m values were determined from double-reciprocal plots (V^{-1} against S^{-1}).

ddC phosphorylation was determined on cell-free high-speed supernatants and intact mitochondria. Briefly, cells were resuspended in 9 volumes of 220 mM mannitol, 70 mM sucrose, 0.2 mM EDTA, 2 mM Hepes, pH 7.4 (homogenizing buffer), and homogenized by a Dounce glass homogenizer. The homogenate was centrifuged first at 1500 *g* to pellet unbroken cells and nuclei, then at 12000 *g* to obtain the mitochondrial fraction; this supernatant was finally centrifuged at 105000 *g* to obtain the cell-free high-speed supernatant. All centrifugations were at 4 °C for 30 min. Protein content was determined by Bradford's protein assay reagent (Bio-Rad).

RESULTS

Delayed cytotoxicity and mitochondrial DNA content

U937 cells grew normally in the presence of 0.25–1 μM ddC for 4–5 days (Figure 1a). After this time, cell growth was inhibited and cells died in a couple of weeks, as has already been reported for Molt-4F cells [5]. Total cellular DNA was extracted from U937 at day 2, 5, 9, 13 and 17 after ddC addition. The DNA was digested with the restriction enzyme *Bam*HI, which linearizes the

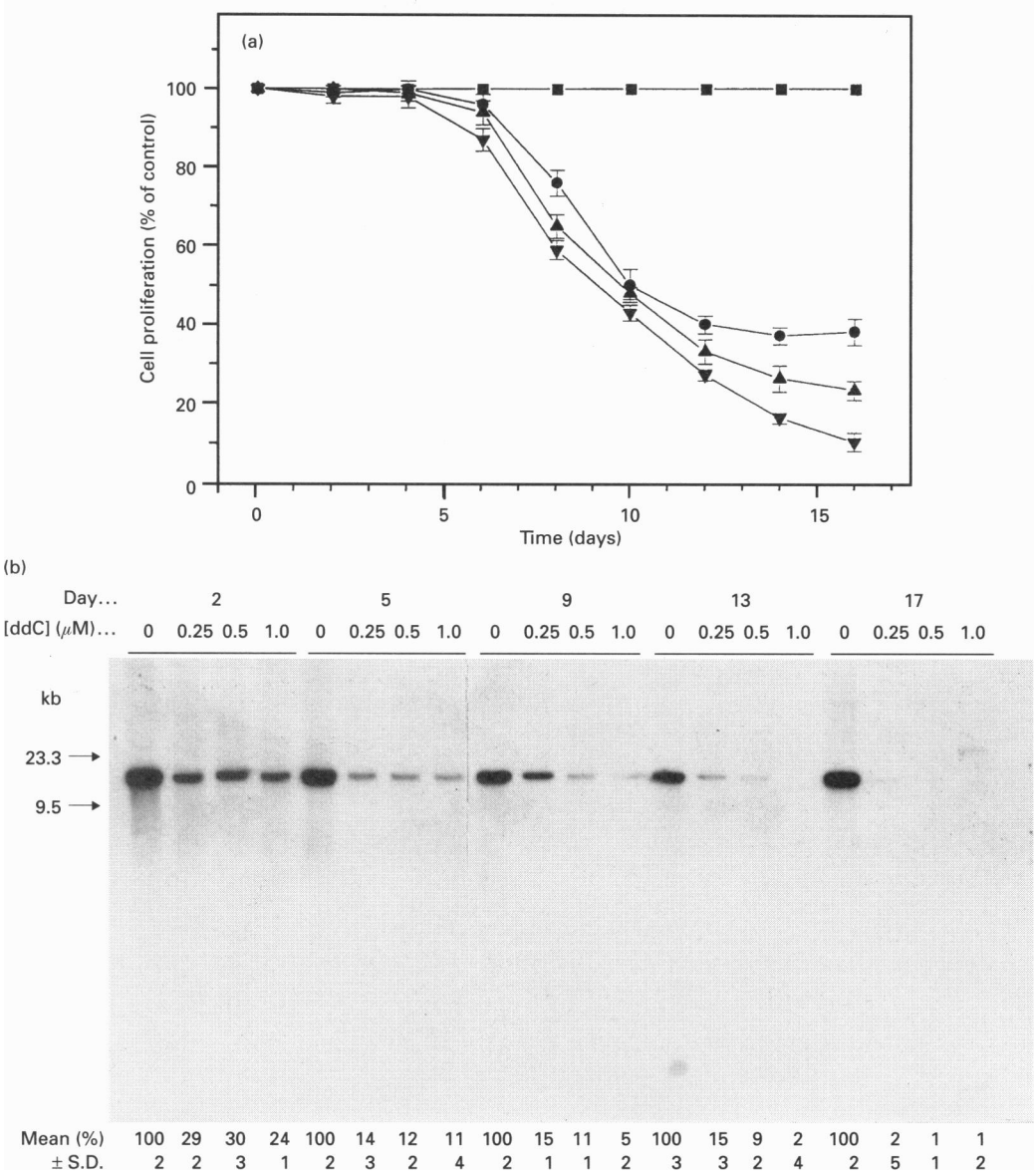

Figure 1 Effect of ddC on cell growth and mitochondrial DNA content

U937 cells were seeded at 10^6/ml and grown in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum, 100 U/ml penicillin G and 100 μg/ml streptomycin. At the times indicated the number of living cells was counted in duplicate with the Trypan Blue dye exclusion test (**a**) and one-half of the cell suspension removed and replaced with fresh medium containing the same drug concentration. Means ± S.D. of five experiments. (**b**) Total cellular DNA was extracted from cells exposed to ddC for different periods. Samples of DNA (0.6 μg, corresponding to about 3×10^5 cells) were digested with the restriction enzyme *Bam*HI, analysed on 0.8% agarose gel and transferred onto a nylon membrane. Detection was with a ^{32}P-labelled 1544 bp human mDNA probe. Figures at the bottom of the mDNA lanes are means ± S.D. percentage of the densitometric areas measured by an LKB laser scan densitometer in five experiments. ■, Control cells; ●, 0.25μM ddC; ▲, 0.5 μM ddC; ▼, 1 μM ddC.

circular mDNA, and analysed by agarose gel electrophoresis and transferred onto a nylon membrane. The mDNA content showed a marked decrease after ddC treatment that was progressive and dependent on ddC concentration and time (Figure 1b), in agreement with the hypothesis [5] that ddC reduces mitochondrial DNA content leading to delayed cytotoxicity.

Induction of resistance to ddC cytotoxicity

Treatment of U937 cells with 0.1 μM ddC resulted in growth inhibition for approximately 3 weeks, after which the cells started to grow again with a proliferative index that was 70 ± 3% that of untreated cells (Figure 2a). mDNA analyses showed a marked decrease in mDNA content at day 6 after treatment, followed by a recovery up to 66 ± 2% of control at day 32 (Figure 2b). This mDNA content was maintained for months even when 0.1 μM ddC was kept in the cell culture medium (at the time of writing we have U937 cells that have been growing in the presence of 0.1 μM ddC for 18 months). In contrast, U937 cells cultured in the presence of 1 μM ddC stopped growing and died (Figures 2a and 2b).The presence of functioning mitochondria was investigated by flow cytometry with the cell-permeant fluorescent dye Rh-123, which is readily sequestered by living mitochondria [17]. U937 cells obtained from an

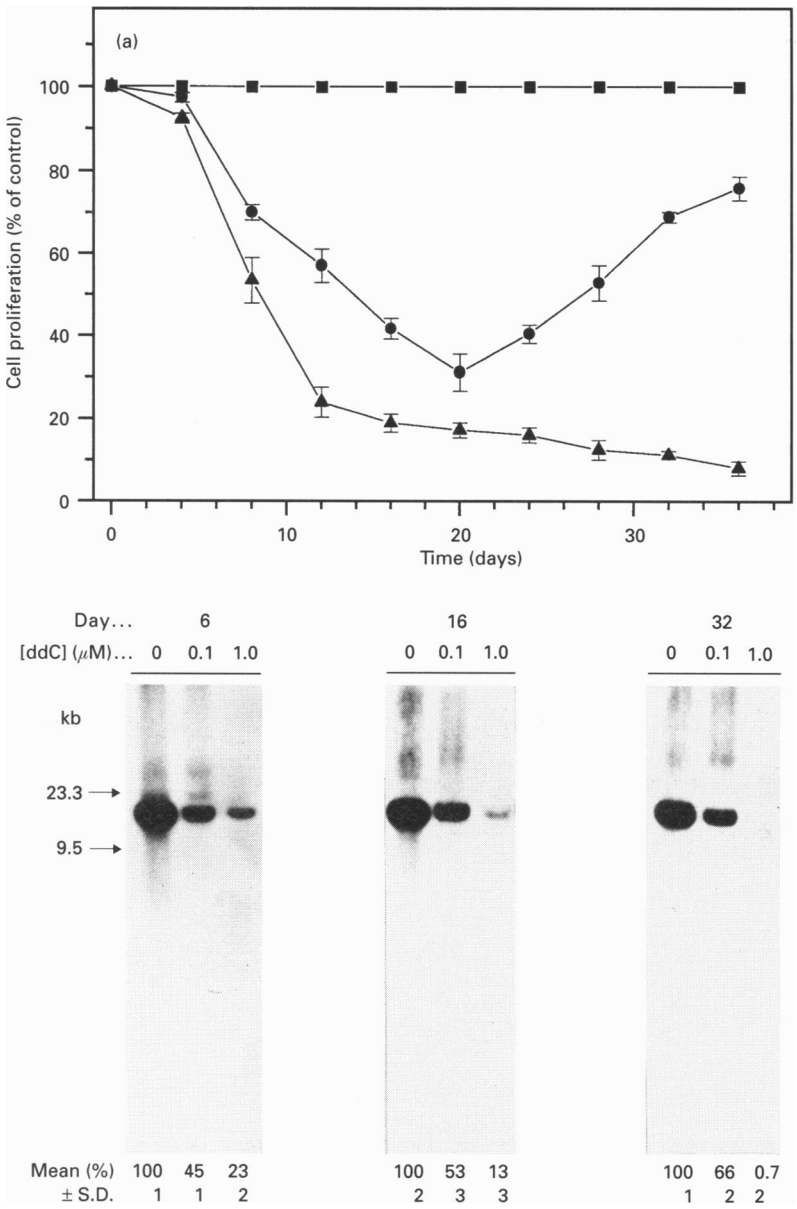

Figure 2 Induction of resistance to ddC in U937 cells

(**a**) Selection of U937 cells resistant to ddC after long-term exposure to the drug. Cells were seeded, grown and counted as in Figure 1. (**b**) Mitochondrial DNA content was measured after *Bam*HI digestion and 0.8% agarose gel electrophoresis of a ^{32}P-labelled mDNA probe. Figures at the bottom the lanes represent means ± S.D. percentage of the densitometric areas evaluated by laser scan densitometry in three experiments. ■, Control cells; ●, 0.1 μM ddC; ▲, 1 μM ddC.

experiment such as that shown in Figure 2, 22 days after ddC addition, were stained with Rh-123. As shown in Figure 3, although almost all the cells are highly fluorescent in the absence of ddC, most are no longer stained if maintained in the presence of 1 μM ddC. An intermediate situation was found when 0.1 μM ddC was used. The kinetics of the process (Figure 3b) clearly show that after 4–5 weeks in the presence of 0.1 μM ddC most of the cells became stained by the dye, whereas most cells at 1 μM ddC died and the few remaining did not recover the initial fluorescence. The results shown in Figure 4 demonstrate that the cells that are selected for their resistance to 0.1 μM ddC toxicity have indeed acquired resistance to the drug. U937 cells, after 2 months of growth in the presence of 0.1 μM ddC (U937-R), were cultured for a further month in the presence of 1 μM ddC. Normal U937 cells died after 2–3 weeks at this drug concentration (see above), whereas U937-R cells continued to grow with a proliferative index 50 ± 3% of that of controls, contained a higher mDNA content than controls, and stained normally with Rh-123 (Figure 4).

ddC metabolism in U937-R cells

To examine the role of ddC metabolism in the resistance of U937 cells to ddC toxicity we have exposed U937 and U937-R cells to a range of [^{3}H]ddC concentrations from 0.1 to 10 μM for 48 h. During this time cell growth was not affected (not shown). The

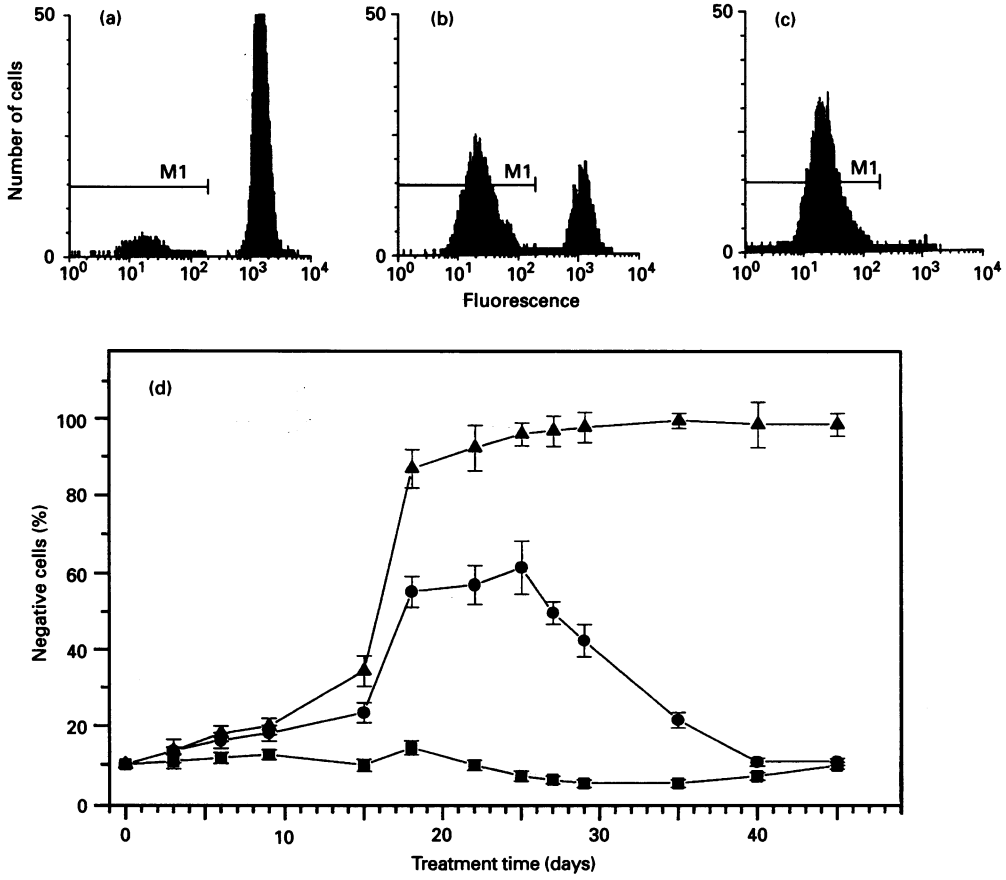

Figure 3 Rh-123 fluorescence of U937 cells grown in the presence of ddC

(**a**–**c**) Fluorescent histograms of cells cultured in the absence (**a**) and in the presence of 0.1 μM (**b**) or 1 μM (**c**) ddC for 22 days. (**d**) Time course of the percentage of fluorescent-negative cells during three experiments (means ± S.D.). ■, Control cells; ●, 0.1 μM ddC; ▲, 1 μM ddC.

uptake of ddC and formation of its phosphorylated metabolites was higher in U937 than in U937-R cells (Figure 5a), although the incorporation of ddC into nucleic acid was similar (Figure 5b), suggesting that delayed cytotoxicity of ddC may not be due to incorporation of the drug into nuclear DNA. When the acid-soluble fractions of U937 and U937-R cells incubated for 24 h with 10 μM [^{3}H]ddC were analysed by ion-pair HPLC chromatography (Figure 6), four major peaks of radioactivity were found. The metabolite eluted at 17 min was in a position identical to authentic ddCTP. ddC, ddCMP and ddCDP eluted at 9.5–12 min and could not be measured individually in the fractions collected at the exit of the wavelength detector. Metabolites of ddC that eluted at 4 and 7 min respectively were tentatively identified as ddCDP-choline and ddCDP-ethanolamine respectively on the basis of the elution positions of dCDP-choline and dCDP-ethanolamine and by the data obtained in other laboratories [18,19]. It is worth noting that the ddCTP concentration in U937-R was 45 % of the controls and that both dideoxyliponucleotides were also markedly reduced. To determine whether these differences in ddC metabolites are peculiar properties of the ddC-resistant cells, we repeated the determinations of Figure 6 at several time points from 1 to 48 h after ddC addition. As shown in Figure 7, the rates of ddCTP and dideoxyliponucleotide formation were significantly reduced in the U937-R cells compared with controls.

Recent reports [13,20,21] have suggested that dideoxynucleotide anabolism is an important determinant of therapeutic effects of pyrimidine nucleoside analogues. Thus we measured the rates of catabolism of ddCTP and dideoxyliponucleotides in U937 and U937-R. As shown in Figure 8, the rates of ddCTP catabolism were similar (1.75 and 1.18 pmol/h per 10^6 cells) in both cell lines, whereas the dideoxyliponucleotides showed a remarkable stability.

ddC influx in U937 and U937-R cells

The influx of ddC in both cell lines was not concentrative (not shown). ddC influx was linear for approximately 2 min at 37 °C and showed no evidence of saturation in the range 1–500 μM (Figure 9). Thus differences in ddC transport cannot explain the acquired resistance of U937-R to ddC toxicity.

dC kinase

Both cytoplasmic deoxycytidine kinase and mitochondrial deoxypyrimidine kinase have been reported to phosphorylate ddC to ddCMP [7]. Preliminary studies performed on intact, isolated mitochondria incubated with ddC have not revealed any detectable ddC phosphorylation (not shown), in agreement with previous reports [6]. In contrast, ddC is actively phosphorylated

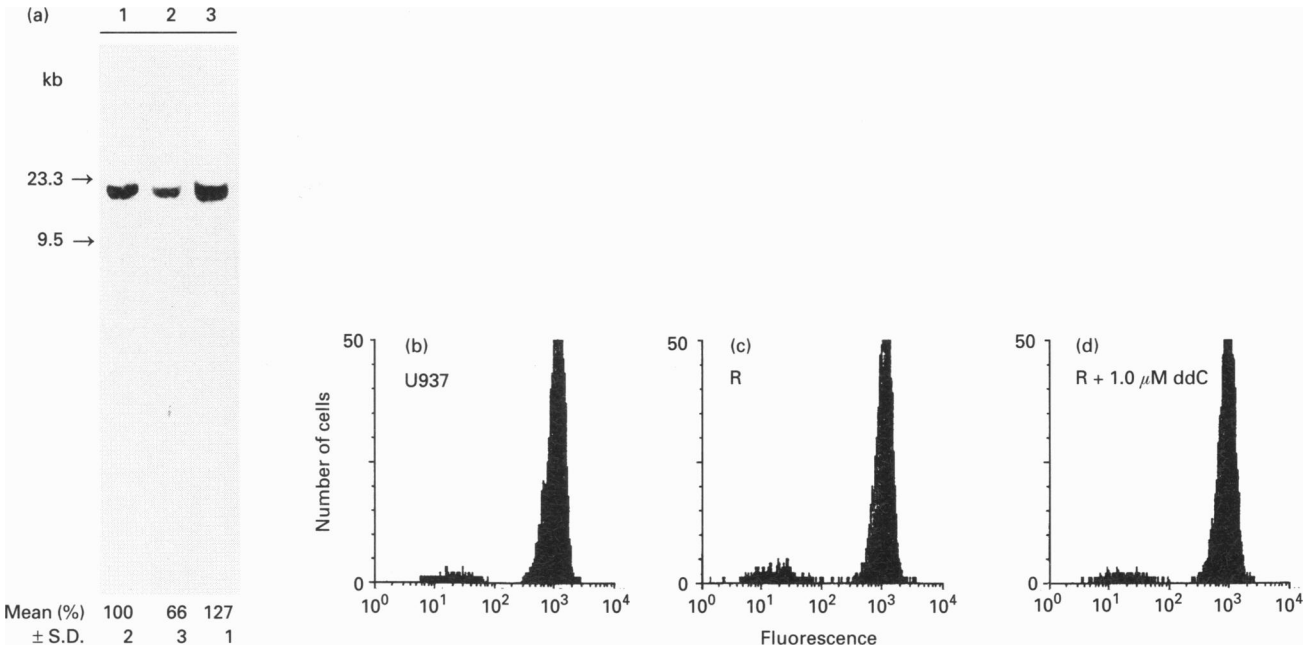

Figure 4 Acquired resistance of U937 cells to ddC

(**a**) Mitochondrial DNA content of U937 (lane 1), U937-R (lane 2) and U937-R cells cultured for 1 month in the presence of 1 μM ddC (lane 3). Total cellular DNA extraction, digestion and analysis was as in the legend of Figure 2. In each case the DNA from 3×10^5 cells was used. (**b–d**) Rh-123 fluorescence of U937 (**b**), U937-R (**c**) and U937-R (**d**) cells grown for 1 month in the presence of 1 μM ddC.

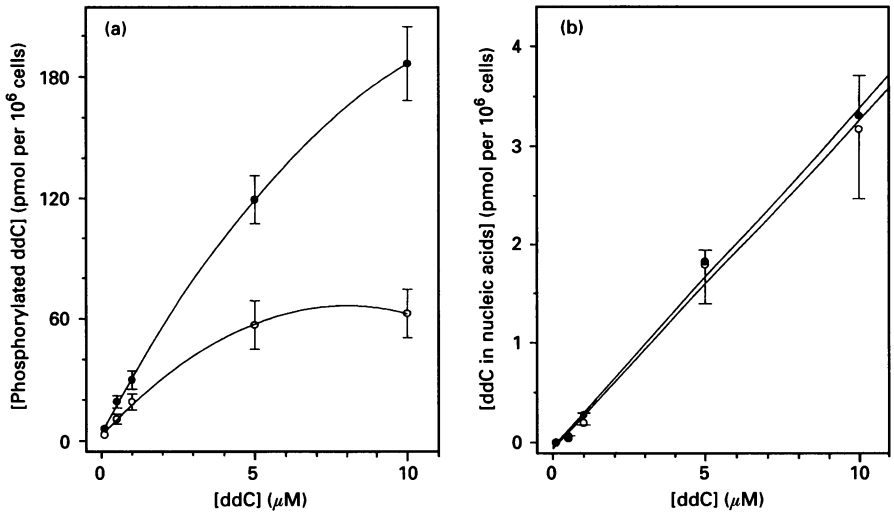

Figure 5 [5,6-^{3}H]ddC incorporation in phosphorylated metabolites and nucleic acids in U937 (●) and U937-R (○) cells

Cells were incubated in the presence of increasing ddC concentrations as indicated, for 48 h at 37 °C in 5% (v/v) CO_2. The amount of drug in (**a**) phosphorylated metabolites (trichloroacetic acid soluble and retained by DE-81 discs) and (**b**) nucleic acids (trichloroacetic acid precipitable) were determined as described in Experimental Procedures. The results are means ± S.D. of three determinations.

by cytosolic supernatants from high-speed centrifugation obtained both from U937 and U937-R. We therefore investigated the kinetic properties of cytoplasmic deoxycytidine kinase from both cell lines. As shown in Table 1, the enzyme from U937-R cells had a decreased affinity and a smaller V_{max} for ddC compared with the enzyme from U937 cells. A similar reduced affinity was found when dC was used as a substrate, with K_m values of 13.67 ± 1.65 μM for U937 cells and 65.50 ± 5.50 μM for U937-R cells. However, V_{max} values were 13.20 ± 0.93 and 50.50 ± 1.5 pmol/min per mg of protein, respectively.

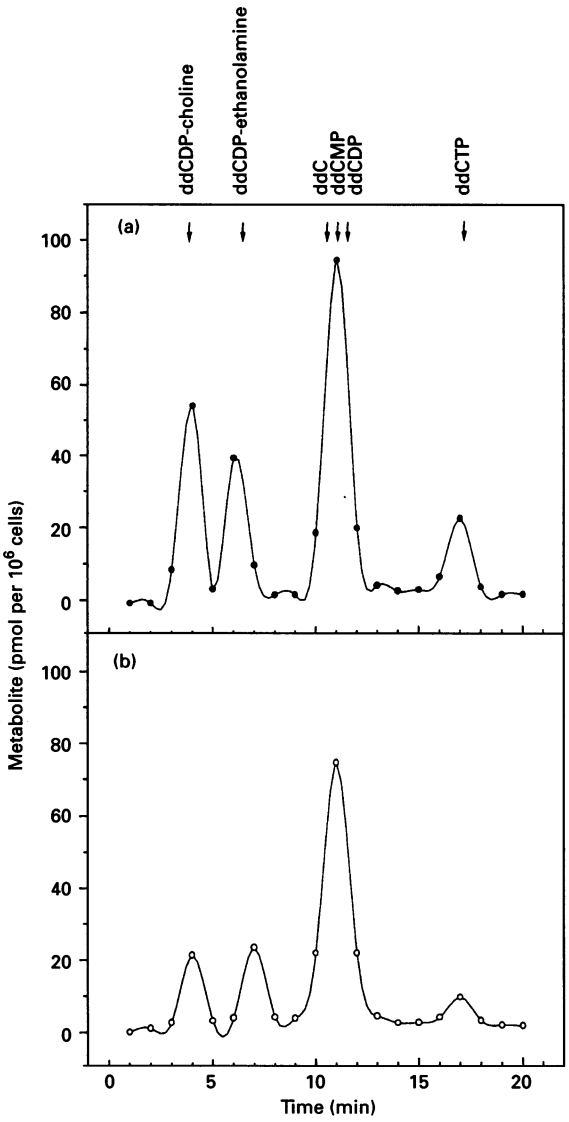

Figure 6 HPLC analysis of [5,6-^{3}H]ddC metabolites in U937 (a) and U937-R (b) cells

Acid-soluble extracts were prepared from 10^6 cells treated for 24 h with 10 μM ddC (597296 c.p.m. per nmol) and analysed by ion-pair HPLC chromatography as described in Experimental Procedures. Fractions of 0.3 ml were collected at the exit of the wavelength detector and counted in a liquid scintillation counter. The retention times of ddCTP, ddC, ddCMP and ddCDP were 17.0, 9.5, 10.5 and 11.5 min, respectively, so it was not possible to quantify ddC, ddCMP and ddCDP correctly under the experimental conditions used.

DISCUSSION

A number of RT inhibitors with straightforward efficacy *in vitro* in inhibiting HIV infectivity are currently approved for use in treatment for HIV. Unfortunately, existing therapies based on RT inhibitors fail to block completely the clinical progression of the disease and spread of the virus (for reviews see [8,22]). One possible explanation for this failure may be the inability to maintain adequate drug levels at the site of viral replication over months or years of therapy. In fact, it should be kept in mind that to be active as RT inhibitors the nucleoside analogues (such as AZT, ddC and ddI) must be phosphorylated to the corresponding 5′-triphosphate derivatives [2] by cellular kinases. However, in many cases, the phosphorylated nucleoside analogues are also cytotoxic [1], so the cells may develop specific drug-resistance mechanism(s). The mechanisms of decreased drug efficacy due to induction of resistance in treated cells are well known for the antitumour nucleoside analogues [23]. Nothing is known about the possibility that similar mechanism(s) can also apply to the antiviral compounds of the dideoxynucleoside family.

In this paper we report that when cultured in the presence of 0.1 μM ddC (a therapeutically relevant concentration), U937 human monoblastoid cells develop resistance to ddC. U937-R cells have a reduced ability to accumulate both ddCTP and dideoxyliponucleotides. This reduced anabolic activity is not caused by a defect in the influx of ddC in U937-R but by altered properties of the cytosolic deoxycytidine kinase. This conclusion seems to be supported by direct evidence that U937-R cells contain an enzyme with a reduced affinity for ddC, a smaller V_{max} and a decreased V_{max}/K_m ratio. The affinity of the enzyme for dC was also diminshed but its V_{max} was higher than in control cells and thus the V_{max}/K_m ratio was much less affected than in U937-R cells. Furthermore previous studies [6] on CEM/araC (a dC kinase-deficient cell line isolated for resistance to araC) have clearly shown that dC kinase-deficient cells are highly resistant to ddC mitochondrial toxicity. We have also noted that U937-R cells contain more mitochondria than normal cells (not shown). However, the mitochondrial DNA content per cell is slightly lower than in controls (Figure 2). Thus it may be that both this reduced ability to accumulate ddC phosphorylated derivatives and the increased number of mitochondria per cell contribute to the mechanism of drug resistance in U937-R. The results reported in this paper also provide support to the mechanism of ddC toxicity suggested by Cheng [1,6]. In fact, both ddC cytotoxicity and development of drug resistance are paralleled by a respective decrease and increase in the mitochondrial DNA content of U937 cells. Furthermore this cytotoxicity, which involves mitochondria, is not due to ddC phosphorylation in the mitochondrial compartment, but rather to cytosolic enzymes. Having shown that ddCTP is produced in the cytosol [6], it remains to be investigated how this nucleotide analogue can penetrate the mitochondrial membrane. Although a ddCTP carrier has not yet been isolated, several lines of evidence suggest its existence. In fact, the metabolites of ddC, but not ddC itself, have been reported to inhibit mDNA synthesis in isolated mitochondria [6], cytoplasmic dC kinase-deficient cells are highly resistant to mitochondrial toxicity by ddC [6], and nucleoside analogues structurally and metabolically similar to ddC (i.e. 2′,3′-dideoxy-3′-thiacytidine, 2′,3′-dideoxy-L-5-fluorocytidine and 2′,3′-dideoxy-L-cytidine) show no inhibition against mitochondrial DNA synthesis because they are not transported into the mitochondria [1,24]. The identification of a carrier for ddCTP would prove useful in searching for possible inhibitors that might prevent the mitochondrial toxicity of ddC.

As shown in Figure 5(b), some ddC is incorporated into nucleic acids. Starnes and Cheng [7] have already reported that ddC is incorporated into DNA. Incorporation of ddC into nucleic acids may be responsible for the inhibition of cell growth at high ddC concentrations (10–500 μM), as usually observed in short-term (2–3 days) exposure of cells to ddC [12]. At lower ddC concentrations, cells probably activate repair mechanisms for the removal of this nucleotide analogue from nuclear DNA. DNA polymerase γ also has an associated 3′ → 5′ exonuclease activity. However, DNA polymerase β (the enzyme responsible for nuclear DNA repair) is inhibited by ddCTP with a K_i of 1.2 ± 0.2 μM, whereas DNA polymerase γ is inhibited by ddCTP with a K_i of

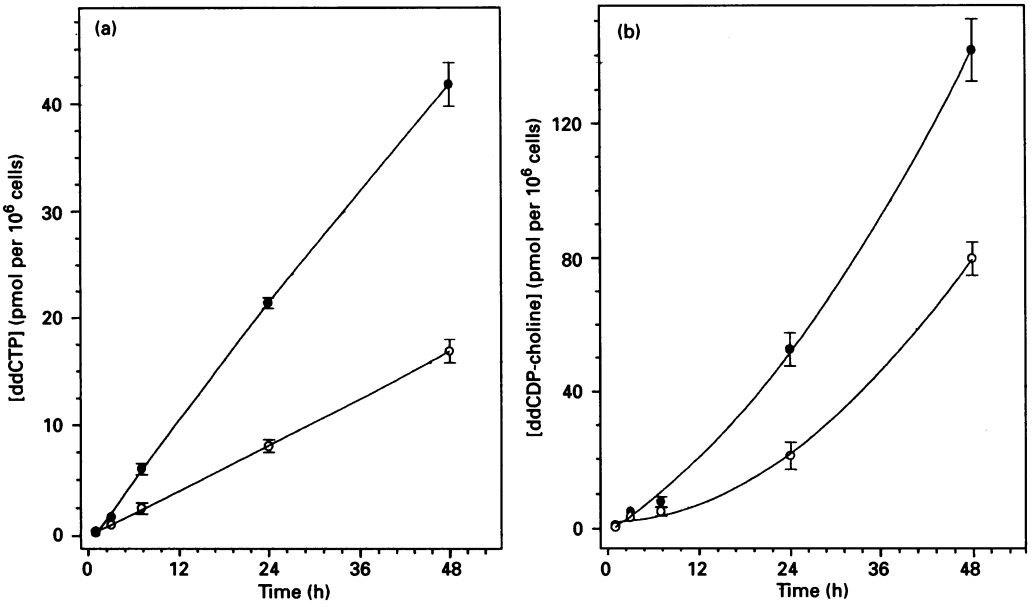

Figure 7 Time course of formation of ddCTP (a) and ddCDP-choline (b) in U937 (●) and U937-R (○) cells

Cells were treated with 10μM [^{3}H]ddC for different times. Acid-soluble extracts were neutralized and analysed by ion-pair HPLC chromatography. Fractions collected at the exit of the wavelength detector were counted in a liquid scintillation counter and peaks corresponding to ddCTP (retention time 17.0 min) and ddCDP-choline (retention time 4.0 min) plotted. Values are means ± S.D. of three determinations.

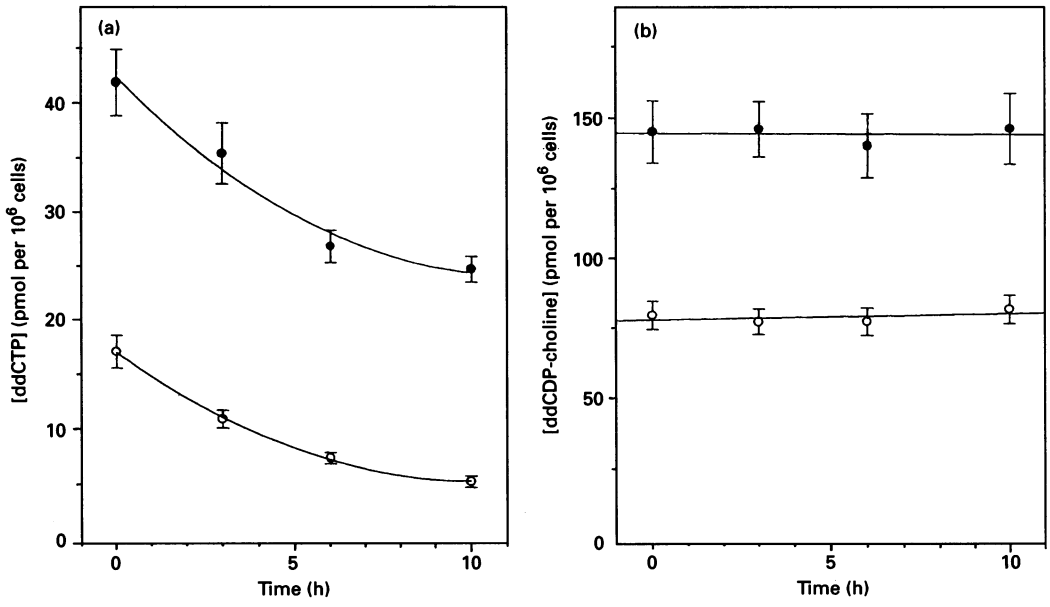

Figure 8 Time course of catabolism of ddCTP (a) and ddCDP-choline (b) in U937 (●) and U937-R (○) cells

Cells were incubated for 48 h in the presence of 10μM [^{3}H]ddC at 37 °C and 5% (v/v) CO_2. The cells were then pelleted, washed and resuspended in fresh medium for the indicated times. Acid-soluble extracts were prepared and analysed as detailed in the legend to Figure 6. Results are means ± S.D. of three experiments.

0.015 ± 0.003 μM [25]. Thus the enzyme responsible for nuclear DNA repair is less sensitive to ddCTP inhibition than mitochondrial DNA polymerase γ [26].

At present it is not known whether the mechanism of induced ddC resistance is also operating *in vivo* in patients undergoing long exposures to ddC. However, the data reported in this paper strongly suggest that ddC metabolism should be maintained in such patients after few months on ddC therapy. If ddC resistance is developed, alternative RT inhibitors, whose activation depends on different cellular enzymes, should be considered.

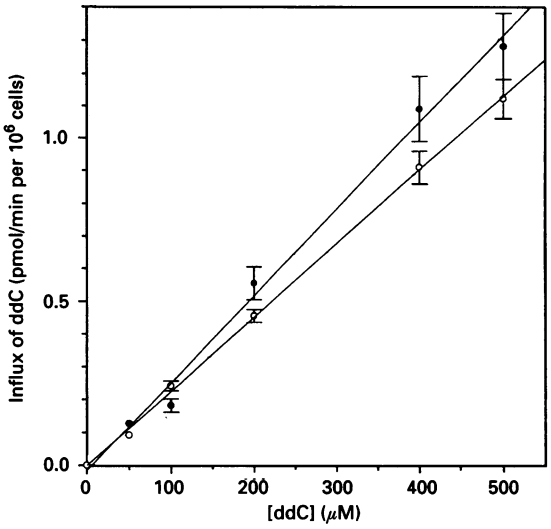

Figure 9 ddC influx in U937 (●) and U937-R (○) cells

Assays were performed at 37 °C in 1.5 ml polypropylene microcentrifuge tubes using an 'oil-stop' method as in [12]. Initial velocities were determined during the first 2 min, when the rate of influx was linear. Initial velocities were determined over a range of ddC concentrations from 1 to 500 μM. Results are means ± S.D. of at least three experiments.

Table 1 Kinetic properties of ddC kinase

All determinations were performed on cell-free high-speed supernatants, at 37 °C and are the means ± S.D. of at least three determinations. K_m values were obtained from double-reciprocal plots (V^{-1} against S^{-1}).

	K_m (μM)	V_{max} (pmol/min per mg of protein)	Substrate efficiency (V_{max}/K_m)
U937	80 ± 7	12.0 ± 1.0	0.15
U937-R	140 ± 9	7.8 ± 0.5	0.056

Received 9 May 1995/4 July 1995; accepted 12 July 1995

This work was supported by Ministero della Sanità, Istituto Superiore di Sanità, Progetto AIDS 1994, Rome, Italy.

REFERENCES

1 Parker, W. B. and Cheng, Y. C. (1984) J. NIH Res. **6**, 57–61
2 Mitsuya, H. and Broder, S. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 1911–1915
3 Yarchoan, R. et al. (1989) Lancet **I**, 76–81
4 Berger, A. R., Arezzo, J. C., Schanmburg, H. H., Skowron, G., Merigan, T., Bozzette, S., Richman, D. and Soo, W. (1993) Neurology **43**, 358–362
5 Chen, C. H. and Cheng, Y. C. (1989) J. Biol. Chem. **264**, 11934–11937
6 Chen, C. H. and Cheng, Y. C. (1992) J. Biol. Chem. **267**, 2856–2859
7 Starnes, M. C. and Cheng, Y. (1987) J. Biol. Chem. **262**, 988–991
8 Johnston, M. I. and Holth, D. F. (1993) Science **260**, 1286–1293
9 Larder, B. A., Darby, G. and Richman, D. D. (1989) Science **243**, 1731–1734
10 Fitzgibbon, J. E., Howell, R. M., Haberzettl, C. A., Sperber, J. J., Gocke, D. J. and Dubin, D. T. (1992) Antimicrob. Agents Chemother. **36**, 155–157
11 Anderson, S. et al. (1981) Nature (London) **290**, 457–465
12 Brandi, G., Rossi, L., Schiavano, G. F., Salvaggio L., Albano, A. and Magnani, M. (1991) Chem. Biol. Interact. **79**, 53–64
13 Ullman, B., Coons, T., Rockwell, S. and McCarton, K. (1988) J. Biol. Chem. **263**, 12391–12396
14 Zimmerman, T. P., Mahony, W. B. and Prus, K. L. (1987) J. Biol. Chem. **262**, 5748–5754
15 Magnani, M., Bianchi, M., Rossi, L. and Stocchi, V. (1989) Biochem. Int. **19**, 227–234
16 Cheng, Y. C., Domin, B. and Lee, L. S. (1977) Biochim. Biophys. Acta **481**, 481–492
17 Johnson, L. V., Walsh, M. L. and Chen, L. B. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 990–994
18 Cooney, D. A., Dalal, M., Mitsuya, H., McMahan, J. B., Nadkarni, M., Balzarini, J., Broder, S. and Johns, D. G. (1986) Biochem. Pharmacol. **35**, 2065–2068
19 Hao, Z. et al. (1993) Drug Metab. Disp. **21**, 738–744
20 Arner, E. S. J., Valentin, A. and Eriksson, S. (1992) J. Biol. Chem. **267**, 10968–10975
21 Arner, E. S. J. and Eriksson, S. (1993) Biochem. Biophys. Res. Commun. **197**, 1499–1504
22 Hirsh, M. and D'Aquila, R. T. (1993) New Engl. J. Med. **328**, 1686–1695
23 Grant, S. (1990) Pharmacol. Ther. **48**, 29–44
24 Lin, T. S., Luo, M. Z., Liu, M. C., Pai, S. B., Dutschman, G. E. and Cheng, Y. C. (1994) Biochem. Pharmacol. **47**, 171–174
25 Martin, J. L., Brown, C. E., Mattews-Davis, N. and Reardon, J. E. (1994) Antimicrob. Agents Chemother. **38**, 2743–2749
26 Lewis, L. and Dalakas, M. C. (1995) Nature Med. **1**, 417–422