Direct analysis of mitochondrial toxicity of antiretroviral drugs

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Objectives: Mitochondrial toxicity is a serious side-effect of antiretroviral drugs, especially nucleoside reverse transcriptase inhibitors (NRTI). An *in vitro* assay to predict mithocondrial toxicity of in-use and developmental NRTI would be invaluable. To test the ability of a cytofluorimetric technique to predict the mitochondrial-dependent pancreatic and hepatic toxicity we used didanosine (ddI) alone or in combination with hydroxyurea (HU).

Methods: The technique is based on the ability of the lipophilic cation JC-1 to enter selectively into mitochondria and change its colour as the membrane potential changes due to toxicity. Mitochondrial toxicity by HU and ddI was evaluated in pancreatic and hepatic human cell lines. The results were expressed as mitochondrial toxicity index (MTI), ranging from 0 to 100: the negative control was 0, and 100 indicating maximal toxicity.

Results: Dose-dependent pancreatic toxicity of ddI was evident after 14 days of culture (MTI 34 $\pm$ 4 at 100 μM, 10 $\pm$ 4 at 10 μM, 2 $\pm$ 3 at 1 μM ddI). HU alone was not toxic (MTI 7 $\pm$ 10 at 100 μM, 2 $\pm$ 2 at 50 μM and 2 $\pm$ 4 at 10 μM HU); however, HU increased the toxicity of high, but not low, concentrations of ddI. For example, the MTI of 10 μM ddI plus 50 μM HU was 54 $\pm$ 9. Negligible mitochondrial toxicity was observed in the hepatic cell line exposed to ddI alone or in combination with HU.

Conclusions: This *in vitro* assay might have *in vivo* relevance. First, ddI-related pancreatitis is dose dependent, and is reported more frequently than hepatic failure, consistent with our *in vitro* results. Second, patients who developed pancreatitis during randomized, controlled trials were treated with HU in combination with 400 mg ddI once daily (high peak concentration of ddI in the blood). In contrast, no pancreatitis was observed when HU was combined with 200 mg ddI twice daily (low peak concentration of ddI). These *in vivo* results are consistent with our *in vitro* observation that HU increases pancreatic cell toxicity in the presence of high concentrations of ddI. The *in vitro* assay described here might be used to predict the mitochondrial toxicity of other NRTI, alone or in combination.

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Introduction

HIV therapy is based on combination regimens, typically including two or more nucleoside reverse transcriptase inhibitors (NRTI) together with one or more protease inhibitors. Mitochondrial toxicity is the common pathway of several NRTI adverse effects [1]. *In vitro* studies have shown that all NRTI inhibit the

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activity of DNA polymerase γ, a key enzyme for mitochondrial DNA (mtDNA) replication [2–7]. The accumulation of mtDNA deficits induces a deficient production of molecules devoted to the intramitochondrial synthesis of adenosine triphosphate (ATP). Once ATP production drops below a certain threshold, sudden mitochondrial and then cellular damage occurs that can lead to cell death [8]. Tissues and organs cannot function properly, and the damage becomes clinically apparent [9,10]. The clinical use of NRTI has been associated with adverse effects caused by mitochondrial dysfunction, such as neuropathy, hepatitis, lactic acidosis, and bone marrow toxicity [11–13]. As hundreds of thousands of patients are treated daily with NRTI, and there is a rich pipeline of new NRTI under development, an assay capable of determining the potential mitochondrial toxicity of drugs or combination of drugs is needed urgently.

To test the validity of an *in vitro* assay for mitochondrial toxicity we chose, as an example, to study the pancreatic toxicity of didanosine (ddI) alone or in combination with hydroxyurea (HU). ddI is known to induce mitochondrial dysfunction in different cell lines [14,15] *in vitro* and pancreatitis *in vivo* [16,17]. Recently, we suggested the use of HU in combination with ddI to inhibit the replication of HIV-1 [18,19]. The mechanism of action of this combination is based upon the ability of HU to inhibit the cellular enzyme ribonucleotide reductase, which catalyses the synthesis of deoxyribonucleoside disphosphates that are precursors of deoxynucleotide trisphosphates (dNTP), the building blocks of DNA. Viral reverse transcriptase uses dNTP to synthesize HIV-1 DNA. ddI inhibits reverse transcriptase by competing with the natural dNTP for incorporation into the viral DNA [19]. By reducing the levels of the natural competitor, HU changes the relative concentration of dNTP and dideoxyATP, thus improving ddI potency [18]. An obvious question is whether HU also potentiates ddI-mediated toxicity.

In recent years, several randomized, controlled clinical trials that showed that HU increases the potency of ddI-containing regimens failed to show any evidence of increased incidence of pancreatic toxicity in the HU-containing arms [20–23]. In contrast, cases of pancreatitis have been described in the course of the most recent ACTG 5025 trial, when ddI was used with and without HU [24]. Pancreatitis is a known adverse event of ddI, and it has been associated with the mitochondrial toxicity of this NRTI [16,17,25–27].

To analyse directly the mitochondrial toxicity of ddI on pancreatic cells and to test whether HU, by decreasing the pool of dNTP and favouring ddI incorporation into mtDNA, can worsen ddI-mediated toxicity, we used a sensitive cytofluorimetric technique based on the staining of intact cells with the lipophilic cationic dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzilmidazolcarbocyanine iodide (JC-1) [28]. This method has been used to study mitochondrial functionality in several conditions [29–33], including primary HIV infection [34,35]. Here, we analysed the *in vitro* effects of ddI and HU, alone or in combination, on the mitochondrial functionality of a pancreatic and an hepatic human cell line.

Materials and methods

Cell lines

BxPC-3, a human pancreatic adenocarcinoma cell line, and SNU-475, a human liver hepatocellular carcinoma cell line (both obtained from ATCC, Manassas, Virginia, USA) were cultured in RPMI-1640, supplemented with 10% heat-inactivated foetal calf serum, 2 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin (all from Gibco BRL, San Giuliano Milanese, Italy) referred to as complete media (CM), in a 75 cm^2 flask (Costar Italia, Milan, Italy) at 37 °C in a humidified atmosphere upplemented with 5% CO_2.

Mitocondrial toxicity assay

BxPC-3 and SNU-475 cells were rinsed with phosphate-buffered saline (PBS, Gibco BRL), and incubated with 0.55% trypsin and 0.2% EDTA (Versene; Eurobio, Les Ulis, Cedex, France) for 5 min at 37 °C. Fresh medium was then added and the viable cells were counted by Trypan blue exclusion. BxPC-3 and SNU-475 cells were aliquoted into one 9.5 cm^2 well of a six-well plate (Costar) at a concentration of 2.5×10^5 and 1.5×10^5 cells/well respectively, with 5 ml of CM and drugs. Stocks of ddI and HU (kindly provided by the National Cancer Institute, Chemotherapeutic Agents Repository, Frederick, Maryland, USA) were dissolved in PBS and stored at −20°C. Four concentrations of ddI (1, 5, 10, 100 μM), three concentrations of HU (10, 50, 100 μM), and nine combinations of the above (except 100 μM ddI) were tested. CM and drugs were refreshed daily. Every 7 days of culture the cells were split, counted and replated with drugs as described. All experiments lasted a minimum of 14 days. At day 14, or every 7 days, 2.5×10^5 cells were harvested and stained with the lipophilic cationic probe JC-1 (Molecular Probes, Eugene, Oregon, USA) as described below, in order to evaluate mitochondrial functionality.

Analysis of mitochondrial membrane potential ($\Delta\psi$) by JC-1 staining

Cells were stained with the $\Delta\psi$-sensitive probe JC-1 as described elsewhere [28]. Briefly, JC-1 is a lipophilic carbocyanine that exists in a monomeric form and is able to accumulate in mitochondria [36,37]. In the presence of a high $\Delta\psi$, JC-1 can reversibly form

aggregates that, after excitation at 488 nm, emit in the orange/red channel (namely, in the FL2 channel of the commonly used flow cytometers). Monomers emit in the green channel (FL1). The collapse in $\Delta\psi$ provokes the decrease in the number of JC-1 aggregates (revealed by a decrease in FL2) and a consequent increase of monomers (an increase in FL1).

BxPC-3 and SNU-475 cells (2.5×10^5) were dispensed into a 5 ml polystyrene round-bottom tube (Falcon, Franklin Lanes, New Jersey, USA) with 1 ml CM supplemented with 10 mM Tris–HCl to maintain a pH of 7.4. The cell suspensions were stained by adding 10 μM JC-1; formation of aggregates was avoided by continuous mixing by vortex during addition of the dye. Cells were incubated for 15 min at room temperature in the dark, washed twice with 2 ml PBS, resuspended in 0.3 ml PBS, and analysed immediately by flow cytometry. Along with the other samples, 2.5×10^5 cells obtained from the untreated sample (negative control) were exposed for 5 min at room temperature to valinomycin (positive control) at a final concentration of 500 nM, washed once with CM containing Tris–HCl, then stained as above. Prior to their use, JC-1 and valinomycin (Sigma Aldrich, Saint-Louis, Missouri, USA) were dissolved in N,N′-dimethylformamide at a concentration of 2.5 mg/ml and at 0.5 mM, respectively. All reagents were pre-warmed at 37 °C before use.

Flow cytometry was performed within 30 min from the end of the staining by using a FACS Vantage SE flow cytometer (Becton Dickinson, San Jose, California, USA) equipped with an argon laser emitting at 488 nm (25 mW). Green fluorescence was measured by a 530 nm/30 nm band pass filter and orange fluorescence was measured by a 575 nm/26 nm band pass filter. The analysis of the samples stained with JC-1 was performed by setting the value of the photomultiplier detecting the signal in FL1 at 340 V and in FL2 at 337 V, both log scale; FL2–FL1 compensation was 24.8% A minimum of 10 000 cells per sample, gated on forward scatter and side scatter graph excluding debris, were acquired in list mode, and the results were analysed with Cell Quest software (Becton Dickinson).

Data analysis

Analysis of the the flow cytometry data was performed by first transforming the results from logarithmic to linear scale, as described previously [38], and then calculating the ratio of the median fluorescence intensity in FL2 divided by the median fluorescence in FL1. The ratios obtained were used to calculate the mitochondrial toxicity index (MTI), which represents the percentage change, assuming the negative control value from each experiment to be equal to 0, and the maximal toxicity to be equal to 100. Negative results were assumed to be equal to zero. Cell counts were expressed as percentages, calculated by assuming that the negative control equaled 100%

Results

In vitro assessment of the MTI of HU and ddI on pancreatic cells

BxPC3 cells were exposed to increasing concentrations of ddI (1, 10, and 100 μM) or of HU (10, 50, and 100 μM) for as long as 28 days. Analysis of mitochondrial functionality and cell counts was performed every 7 days. In the flow cytometric analysis the differences between samples were detected by the shift of the fluorescence toward the green and calculated as described by using the linearized median fluorescence values obtained in FL1 and FL2. Fig. 1 shows a representative example of the results. A shift between the negative control and the valinomycin treated sample (positive sample) was evident. Such a shift was also visible in the sample treated with ddI at high concentration (100 μM). In the time-course study, shown in Table 1, there was negligible mitochondrial toxicity at a low ddI concentration (MTI, 4 ± 5 at day 21 with 1 μM ddI). At 10 μM ddI the MTI was as high as 20 ± 13 at day 21 of culture; however, this effect appeared to be reversible (MTI, 9 ± 5 at day 28), and the cell number decreased moderately (percentage of viable cells, $66 \pm 28\%$ compared to the negative control). At 100 μM ddI, toxicity was evident after 14 days of culture. The MTI was 34 ± 4 and the cell percentage was $17 \pm 4\%$ compared to the negative control. Cell cultures were terminated because of excess toxicity. HU alone did not affect substantially the mitochondrial functionality at any of the concentrations tested (Table 1). However, the cell cultures incubated with 100 μM HU were discontinued after 14 days of culture, because of low cell percentage ($30 \pm 37\%$ compared to the negative control). Unlike ddI, the cytotoxicity of HU was not preceded by mitochondrial toxicity.

Effects of HU on the ddI-induced mitochondrial toxicity on pancreatic cells.

After the first set of experiments, we investigated the effect of HU on ddI-induced mitochondrial toxicity at day 14 of culture. In the pancreatic cell line BxPC3 (Fig. 2, upper panels), the combination of 1 μM ddI plus HU did not affect mitochondrial functionality at any of the concentrations tested. There was an HU dose-dependent decrease in cell number (ranging from $96 \pm 6\%$ with 10 μM HU to $50 \pm 35\%$ with 100 μM HU). The combination of 5 μM ddI plus HU (Fig. 2, middle panels) induced moderate mitochondrial dysfunction that depended on the HU concentration (MTI, 1 ± 2 5 μM ddI alone; MTI, 20 ± 19 with 10 μM HU; MTI, 13 ± 13 with 50 μM HU; MTI,

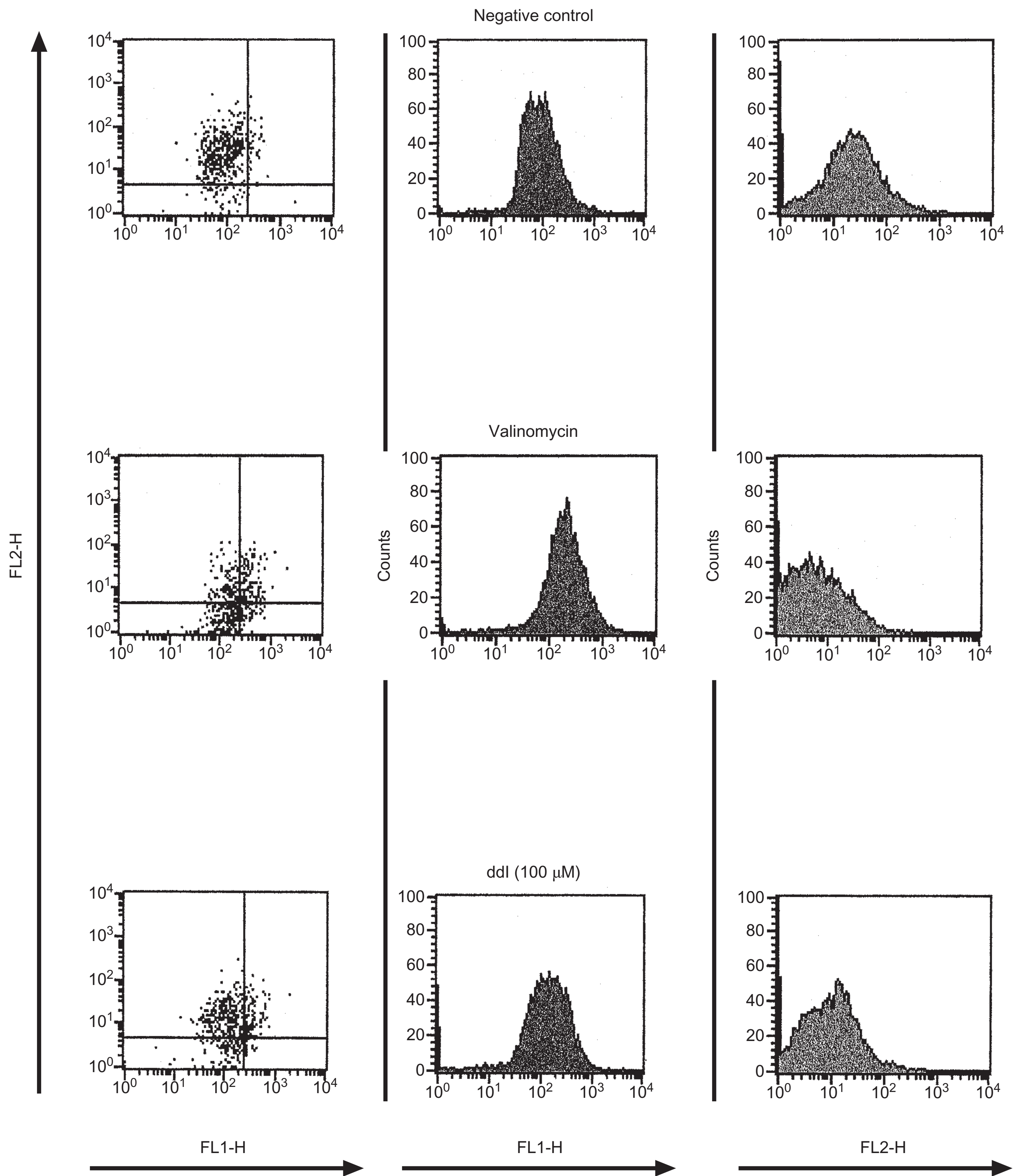

Fig. 1. BxPC-3 cells were cultured for 14 days in the presence of ddI or HU or culture media, and then stained with JC-1 as described. An aliquot of untreated cells (negative control) was exposed to valinomicyn (positive control) before staining. As shown in the dot plot, mitochondrial depolarization induced a shift in JC-1 fluorescence from FL2 to FL1 in the valinomicyn sample as well as in the 100 μM ddI sample. Median fluorescence intensity in the valinomicyn and ddI samples decreased in the FL2 and increased in the FL1, as shown by the FACS histograms. Median fluorescence intensity was used to calculate the MTI (see text).

Table 1. *In vitro* kinetics of the mitochondrial toxicity induced by ddI or HU alone in the pancreatic cell line BxPC-3. Data are expressed as mitochondiral toxicity index (see text) and were obtained the time point indicated in the table. Values are the arithmetic mean of three experiments ± SD.

	ddI 1 μM	ddI 10 μM	ddI 100 μM	HU 10 μM	HU 100 μM
Day 7	1 ± 3	2 ± 2	6 ± 9	2 ± 4	1 ± 1
Day 14	2 ± 3	10 ± 4	34 ± 4	2 ± 4	7 ± 10
Day 21	4 ± 5	20 ± 13	NA	2 ± 3	NA
Day 28	3 ± 4	9 ± 5	NA	5 ± 9	NA

NA, Data not available.

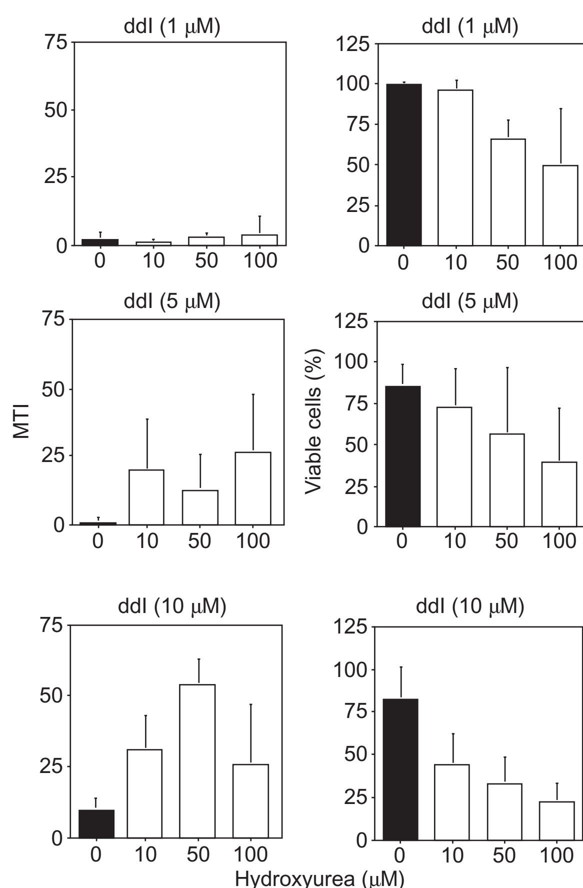

Fig. 2. MTI (left panels) and percentage of viable cells (right panels) after 14 days of culture of the human pancreatic cell BxPC-3. Cells were exposed to three concentrations of ddI (1 μM, upper panels; 5 μM, middle panels; 10 μM, lower panels) alone or in combination with increasing concentrations of HU. MTI was calculated as specified in the text, and the untreated sample (negative control) was assumed to be equal to 0. Cell counts were expressed as percentages, calculated assuming that the negative control equals 100%. Data shown are the arithmetic mean of three different experiments ± SD.

27 ± 21 with 100 μM HU). A dose-dependent decrease in the cell number (73 ± 23% of negative control with 10 μM HU, 57 ± 40% with 50 μM HU, 40 ± 33% with 100 μM HU) was also documented. Finally, ddI at a concentration of 10 μM combined with all concentrations of HU (Fig. 2, lower panels) severely affected mitochondrial functionality (MTI, 31 ± 12 with 10 μM HU; MTI, 54 ± 9 with 50 μM HU; MTI, 26 ± 21 with 100 μM HU). Also in this case the decrease of the cell number correlated with the increase of HU concentration, and not with the MTI values. The toxicity observed at day 14 with the combination of 5 μM or 10 μM ddI plus HU were similar to those obtained with the use of 100 μM ddI alone (MTI, 34 ± 4, Table 1), or with the use of valinomicyn 500 nM (MTI, 38 ± 22; data not shown).

Effects of HU and ddI on hepatic cells

Limited mitochondrial toxicity was observed in the hepatic cell line SNU-475. Increasing concentrations of ddI did not correlate with increasing mitochondrial toxicity (MTI, 5 ± 7 with 1 μM ddI; MTI, 8 ± 11 with 5 μM ddI; MTI, 9 ± 13 with 10 μM ddI). Moreover, the addition of HU in culture in combination with ddI did not further increase mitochondrial toxicity in this cell line (Fig. 3). Furthermore, SNU-475 cell replication was not affected by the presence of HU in culture (data not shown).

Discussion

Drug toxicity is one of the main obstacles to the successful management of antiretroviral regimens [39]. As the vast majority of the combinations presently used is based upon one or more NRTI, a reliable means of predicting the toxicity profile of NRTI or of NRTI-containing combinations would enhance the design of clinical studies and daily clinical management of toxicity. We show here that an *in vitro* assay can be used to predict mitochondrial toxicity of NRTI or NRTI-based combinations.

In our study, ddI concentrations were chosen to reflect the *in vivo* concentrations of the drugs, the C_{max} of ddI being 6.5 μM ± 3.3 and 3.4 μM ± 1.9 with 400 mg once daily and 200 mg twice daily regimens, respectively [40]. The concentrations of HU used here also represent the range of concentrations attainable *in vivo*

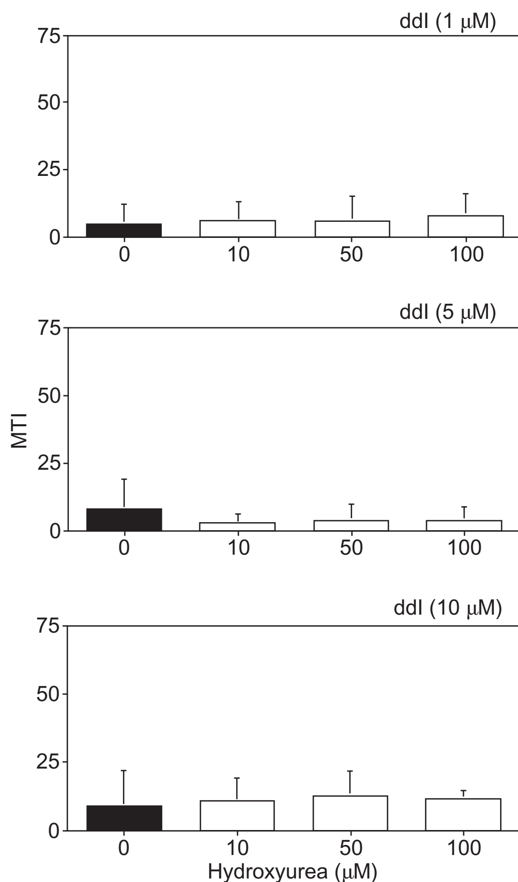

Fig. 3. MTI after 14 days of culture of the human hepatic cell SNU-475 in the presence of ddI (1 μM, upper panel; 5 μM, middle panel; 10 μM, lower panel) alone or in combination with increasing concentrations of HU. MTI was calculated as specified in the text, the untreated sample (negative control) was assumed to be equal to 0. Data shown are the arithmetic mean of three different experiments ± SD.

in HIV-infected patients with the commonly used 500 mg twice daily regimen [41]. The concentration-dependent toxicity of ddI observed in our *in vitro* experiments is consistent with the observation that the pancreatic toxicity is dose dependent *in vivo* [25–27,42]. Conversely, only limited toxicity was noticeable after 14 days of culture in the hepatic cell line SNU-475, at any of the ddI concentrations tested. The difference might be due to a variance in the reserve of mitochondrial capacity of the pancreatic and hepatic cells. Recently, it has been proposed that the mitochondrial reserve in hepatocytes is such that a complete inhibition of mitochondrial function is unlikely [43]. This hypothesis and our data are consistent with the *in vivo* observation that pancreatitis is an adverse effect more frequently reported than hepatic failure in patients treated with ddI [25–27,42].

Although HU alone did not alter mitochondrial functionality, it did inhibit cell division, consistent with the known cytostatic effects of this drug. However, the addition of HU increased the mitochondrial toxicity of high, but not low, concentrations of ddI in the pancreatic cell line (i.e. 100 μM HU plus 5 μM ddI showed the same toxicity parameters as 100 μM ddI). The somewhat large difference of MTI increase after addition of HU to 5 μM ddI and to 10 μM ddI, can be attributed to the fact that mitochondrial dysfunction becomes evident only when ATP production decreases below a certain threshold [8]. Thus, a relatively small increase in ddI concentration in the critical range could have a dramatic effect on mitochondrial functionality. The effect of HU might be explained by the ability of this drug to increase the relative concentration of ddI [18]. However, the cytostatic effect of HU might also play a role in increasing the toxicity of ddI. It has been shown that the total number of mtDNA defects is the main factor in determining clinical expression of the mitochondrial toxicity. Cells with a lower turnover of mtDNA (such as non-dividing or slow-dividing cells) accumulate a larger number of mtDNA defects, thereby increasing the probability of cell damage [44,45]. Therefore, HU, by reducing the number of cell replications, might increase the number of mtDNA defects per cell. These mechanisms are not necessarily mutually exclusive.

These results might be clinically relevant. No pancreatitis has been reported in patients treated with HU monotherapy [21,46,47]. No case of pancreatitis has been described in more than 500 patients (mainly antiretroviral drug naive) enrolled in four randomized, controlled, clinical trials, using ddI plus HU [20–23]. In striking contrast, seven cases of pancreatitis (three in the ddI, stavudine, indinavir arm; and four in the ddI, stavudine, indinavir, HU arm) have been reported among 136 patients in the recent ACTG 5025 trial, and two deaths occurred in the HU-containing arm [24]. Two factors might explain such a high incidence of pancreatic toxicity in this trial. First, the patients had been heavily pre-exposed to drugs including non-nucleoside reverse transcriptase inhibitors, and may have already suffered mitochondrial damage. Second, the patients were treated with ddI in a once daily regimen, and as previously reported, the C_{max} of the ddI once daily regimen is twice that attainable with a twice daily regimen, in some patients as high as 10 μM [40]. It is known that ddI-dependent mitochondrial toxicity is dose dependent [25,27,48,49] and we have shown that HU increases the toxicity of high ddI concentrations. It is possible that the once daily (high C_{max}) regimen facilitated the onset of pancreatitis. If so, it is conceivable that the 400 mg ddI enteric coded formulation will show a favourable toxicity profile, alone or in combination with HU, as the peak concentrations of 400 mg enteric coded ddI and 200 mg ddI twice daily are similar.

One has to notice, however, that the C_{max} value

represents the peak drug concentration, which lasts for a relatively short time (approximately 1 h), whereas in our study drug concentrations were kept constant (and refreshed daily to avoid drug degradation). Therefore, while our *in vitro* assay might predict which drugs could induce toxic effects alone or in combination, it cannot predict the dosage of the drug that will eventually induce or potentiate toxicity *in vivo*.

Another important consideration is that there are individual variances in sensitivity to drugs. This might explain why so few patients develop pancreatitis when treated with ddI. Such differences might be correlated to the interindividual polymorphisms of DNA polymerase γ, because enzymes from different individuals might have different affinities to NRTI incorporation; or alternatively, the threshold expression of the energy deficit might be lower in some individuals [1,50,51]. Moreover, it has been shown that some NRTI increase the levels of mutated mtDNA by decreasing the proliferation of cells containing wild-type mtDNA [51].

Finally, to our knowledge, this is the first study that evaluates directly, at the functional level, the mitochondrial toxicity of a combination of two drugs in human cell lines of hepatic or pancreatic origin. In this study we show that NRTI-related mitochondrial toxicity can be evaluated *in vitro* by using a sensitive JC-1 technique which detects changes in $\Delta\psi$. This test method has distinct advantages over previously reported methods such as mtDNA content, cell viability, lactic acid production, or mitochondrial morphology (for a review see [1]) as those techniques are cumbersome, semi-quantitative and they measure mitochondrial functionality indirectly. The assay described here is quantitative, relatively easy to perform, allows the concurrent analysis of large numbers of samples, measures mitochondrial functionality directly, and could become invaluable to test the potential toxicity of presently used, and especially to-be-used, drugs or drug combinations. It would be very interesting to investigate whether the JC-1 staining technique can be applied *in vivo* to develop a system useful for predicting mitochondrial toxicity, and to test diverse susceptibility of different organs to NRTI-dependent mitochondrial toxicity.

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