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Differential effects of zidovudine and zidovudine triphosphate on mitochondrial permeability transition and oxidative phosphorylation

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1 The effects of zidovudine (ZDV) and zidovudine triphosphate (ZDV-3P) on Ca^{2+}-induced mitochondrial permeability transition (MPT), respiratory control ratio (RCR) and ATP synthesis have been investigated on isolated rat liver mitochondria.

2 ZDV slightly but significantly decreased RCR and ATP synthesis but was ineffective in inhibiting MPT. In contrast, ZDV-3P did not alter RCR and ATP synthesis but strongly inhibited MPT ($IC_{50} = 3.0 \pm 0.9$ μM).

3 The effect of ZDV-3P on mitochondrial swelling required a preincubation time. When incubated 10 min with mitochondria, ZDV-3P (8 μM) totally inhibited the rate of swelling.

4 ADP, ATP and atractyloside, which are agents known to interact with the mitochondrial adenine nucleotide carrier (ANC), antagonized the effect of ZDV-3P on mitochondrial swelling. Indeed, the IC_{50} value of ZDV-3P increased from 3.0 to 17.4, 93.6 and 66.5 μM, in the presence of 20 μM, ADP, ATP or atractyloside, respectively.

5 ZDV-3P did not displace [^{3}H]-ATP from its mitochondrial binding site(s) whereas ADP and atractyloside did, suggesting that ZDV-3P and [^{3}H]-ATP do not share the same binding sites.

6 ZDV-3P did not affect either mitochondrial respiration or ATP synthesis but inhibited Ca^{2+}-dependent mitochondrial swelling. It was concluded that mitochondrial toxic effects observed during the chronic administration of ZDV cannot be related to its active metabolite (ZDV-3P).

Keywords: Zidovudine (ZDV); zidovudine-3P (ZDV-3P); liver mitochondria; swelling; adenine nucleotide carrier (ANC); mitochondrial permeability transition (MPT); ATP; ADP; atractyloside

Introduction

Zidovudine (3′-azido-3′-deoxythymidine, ZDV) was found to be effective against human immunodeficiency virus (HIV) *in vivo*, which has led to its chronic use in patients with acquired immunodeficiency syndrome (AIDS) (Fischl *et al*., 1987). As a prodrug, ZDV is activated in the body by three successive phosphorylation processes yielding ZDV monophosphate, diphosphate and lastly triphosphate (ZDV-3P) (Furman *et al*., 1986). These biotransformations, which occur within the cytosol of host cells, are catalyzed by thymidine kinase, thymidylate kinase and nucleoside diphosphokinase, respectively. It is noteworthy that the antiviral activity of ZDV depends on the ability of host cells to accumulate ZDV-3P which acts as an inhibitor of thymidine triphosphate, blocking the HIV reverse transcriptase effect.

Although well tolerated, long-term administration of ZDV has been shown to induce several side effects including neuromuscular and liver toxicity (Helbert *et al*., 1988; Dalakas & Pezeshkpour, 1988; Chen *et al*., 1992). A cellular target of ZDV toxicity is the mitochondrion (Dalakas *et al*., 1990; Mhiri *et al*., 1991). Indeed histological abnormalities such as numerous and enlarged proliferative mitochondria have been observed (Napolitano, 1993). This corresponds to the formation of a mitochondrial permeability transition (MPT) which suggests an alteration of the membrane impermeability leading to a decrease in adenosine 5′-triphosphate (ATP) synthesis.

MPT is due to the opening of a nonselective channel with a minimum diameter of 2.8 nm allowing diffusion of solutes with molecular mass up to 1500 daltons. The pore is regulated by matrix Ca^{2+} in a reversible manner, as indicated by the pioneering work of Haworth and Hunter (1979). Although a definitive description of the components that constitute the MPT is not yet available, the adenine nucleotide carrier (ANC) was suggested to be specifically involved, because of the observation that specific ligands of the ANC, namely atractyloside (Lapidus & Sokolove, 1992), bongkrekate, ADP (De Macedo *et al*., 1993) as well as ATP (Carbonera & Azzone, 1988), affect the MPT.

As the above subcellular events are easily reproducible *in vitro* in isolated mitochondria (Gunter & Pfeiffer, 1990), we have investigated whether MPT could be either generated and/or inhibited by ZDV and its active metabolite, ZDV-3P. As it was not known whether the prodrug (ZDV) or the active metabolite (ZDV-3P) was responsible for the observed mitochondrial toxicity, we have also compared the effects of both agents on mitochondrial oxidative phosphorylation.

Methods

All animal procedures used in this study are in strict accordance with the French Agency's policies about animal experimentation.

Isolation of mitochondria

Rat liver mitochondria were isolated as described by Johnson and Lardy (1967). Briefly, male Wistar rats weighing approximately 250 to 300g were decapitated, livers were rapidly excised and placed immediately in medium containing 250 mM sucrose, 10 mM Tris and 1 mM EGTA, pH = 7.8 at 4°C. The

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tissue was minced with scissors and homogenized on ice with a Teflon Potter homogenizer. The homogenate was centrifuged at 600 *g* for 10 min (Sorvall RC 28 S). The supernatant was centrifuged for 5 min at 15 000 *g* to obtain the mitochondrial pellet. The latter was washed with the same medium and centrifuged at 15 000 g for 5 min. The mitochondrial pellet was washed with medium from which EGTA was omitted and centrifuged for 5 min at 15 000 g resulting in a final pellet containing approximately 50 mg of protein ml^{-1}. The mitochondrial suspension was stored on ice before the swelling measurements. The protein content was determined by the method of Lowry *et al.* (1951).

Mitochondrial swelling measurements

Mitochondrial swelling was assessed by measuring the change in absorbance at 520 nm by using a Hitachi model U-3000 spectrophotometer. The method of Halestrap and Davidson (1990) was used with some modifications. Mitochondria (4 mg) were added to 3.6 ml of buffer containing 150 mM sucrose, 5 mM Tris, 0.5 μg of rotenone ml^{-1} at pH 7.4 and 25°C; 1.8 ml of this suspension was added to both sample and reference cuvettes in the presence or absence of different inhibitors. After 6 min of incubation at 25°C, 50 μM $CaCl_2$ were added to both cuvettes. Four minutes later the swelling was initiated by the introduction of 10 μM *tert*-butylhydroperoxide (*t*-BH) to the sample cuvette only and the A_{520} scanning was started.

Membrane binding experiments with [^{3}H]-ATP

Binding of [^{3}H]-ATP was carried out in a total volume of 500 μl containing 400 μl of either intact or sonicated mitochondria (0.1 mg ml^{-1}), 2 nM [^{3}H]-ATP and different concentrations of competing drug or buffer for 4 min at 25°C. Specific binding was defined as the difference between total binding and binding in the presence of 0.3 mM ADP.

Measurements of mitochondrial respiration parameters

O_2 consumption was measured by a Clark type oxygen microelectrode in a thermostat-controlled chamber. Mitochondrial respiration (1.5 mg protein ml^{-1}) was initiated by addition of succinate (6 mM final concentration), and oxidative phosphorylation was initiated by addition of ADP to a final concentration of 0.1 mM. O_2 consumption recordings allowed the calculation of the respiratory control ratio (RCR), V_3 which is the rate of state 3 (ADP stimulated) respiration and the P/O ratio (ADP used divided by oxygen (O_2) consumed in state 3 respiration).

Drugs used

ZDV-3P was kindly supplied by Glaxo-Wellcome Laboratories. [^{3}H]-ATP (46 Ci $mmol^{-1}$) was purchased from New England Nuclear. ZDV, ATP, ADP and atractyloside were obtained from Sigma. All other chemicals were purchased from standard suppliers and were of the highest purity commercially available.

Data analysis and statistics

The initial rate of swelling (V_i) was expressed as change in absorbance units min^{-1} mg^{-1} protein and the percentage of swelling inhibition rate (E) induced by different inhibitors was determined as follows:

$$E = \frac{V_{max} - V_i}{V_{max}} \times 100 \qquad (1)$$

where V_{max} is the maximal swelling rate in the absence of the inhibitor.

Data of swelling inhibition experiments were fitted to the following equation:

$$E = \frac{E_{max} \cdot C^{n_H}}{IC_{50}{}^{n_H} + C^{n_H}} \qquad (2)$$

Where E is the swelling inhibition rate (as %) in the presence of a particular drug concentration (C), E_{max} the maximal effect, IC_{50} the concentration that inhibits 50% of the maximal effect and n_H the pseudo-Hill coefficient (Weiland & Molinoff, 1981).

For the binding experiments the following equation was used:

$$B = \frac{B_{max} \cdot IC_{50}{}^{n_H}}{IC_{50}{}^{n_H} + C^{n_H}} \qquad (3)$$

Where B is the number of binding sites observed in the presence of a particular inhibitor concentration (C), B_{max} the maximal number of a binding sites, IC_{50} the concentration that inhibits 50% of the maximal binding and n_H the pseudo-Hill coefficient.

All parameters were calculated by means of a non-linear regression analysis by use of a commercially available software (Micropharm INSERM 1990, Urien, 1995).

Statistical comparisons were made between two parameters by means of Student's two tailed unpaired *t* test. A *P* value <0.05 was considered statistically significant. All values are shown as means $\pm$ s.d. of three different experiments.

Results

Comparison of the effects of ZDV and ZDV-3P on mitochondrial swelling

MPT occurrence was assessed by the resulting large amplitude swelling of these organelles. Figure 1 (line e) shows that when mitochondria were exposed to Ca^{2+} (50 μM) and *t*-BH (10 μM) they swelled, as shown by the time course decrement of mitochondrial absorbance at 520 nm. ZDV-3P (3 μM) inhibited, by approximately 50%, the rate of this motochondrial swelling (line c). However ZDV up to a concentration of 100 μM failed to inhibit this rate (line d). In the absence of Ca^{2+} and *t*-BH, neither ZDV nor ZDV-3P affected mitochondrial volume (lines a and b). Phosphate compounds are known to chelate divalent cations. In fact, when ZDV-3P was introduced in the cuvette at the same time as Ca^{2+}, its inhibitory effect did not occur. This can be explained by the chelation of Ca^{2+} by ZDV-3P as well as other polyphosphates which prevent Ca^{2+} binding to mitochondria. However, when ZDV-3P was added to mitochondria before Ca^{2+}, ZDV-3P had time to interact with these organelles and its inhibitory effect was observed. Moreover, under our experimental conditions, the presence of another divalent cation, Mg^{2+}, did not affect the effect of ZDV-3P (results not shown) when the order of additions was respected, i.e. mitochondria followed by ZDV-3P and then the cation.

Time- and concentration-dependences of ZDV-3P effect

Before the induction of mitochondrial swelling, we preincubated mitochondria in the absence and presence of ZDV-3P for various times ranging from 0 to 10 min. Figure 2 shows that the inhibitory effect of ZDV-3P increased when the preincubation time was increased. Indeed, ZDV-3P failed to affect the rate of the mitochondrial swelling when no preincubation was performed (i.e. t = 0 min). However when the incubation time was 10 min, ZDV-3P (8 μM) totally inhibited this rate. Furthermore, the inhibitory effect of ZDV-3P was concentration-dependent, as illustrated by the concentration-effect curve depicted in Figure 3. The IC_{50} for ZDV-3P in inhibiting the rate of this swelling was 3.0 ± 0.9 μM.

Interactions of ZDV-3P with ADP, ATP and atractyloside

ADP, ATP and atractyloside are molecules known to interact with the ANC (Le Quoc & Le Quoc, 1988). Many investigators have reported that the latter might be a component of MPT (Toninello *et al*., 1983; De Macedo *et al*., 1993). This has led us to investigate whether the above effectors of ANC could affect the inhibitory effect of ZDV-3P on mitochondrial swelling. Figure 4 shows that both ATP and atractyloside were more potent than ADP in antagonizing the effect of ZDV-3P. Indeed, 20 μM of either ATP or atractyloside increased the IC_{50} of ZDV-3P from 3.0 ± 0.9 μM to 93.6 ± 3.33 μM ($P < 0.001$) and 66.5 ± 6.40 μM ($P < 0.005$), respectively, whereas 20 μM ADP increased this value to 17.4 ± 0.40 μM ($P < 0.002$). Under our experimental conditions, it was verified that 20 μM ADP, ATP or atractyloside did not affect mitochondrial swelling induced by *t*-BH in the presence of Ca^{2+}. Since both ZDV-3P and ATP are nucleoside triphosphate analogues, we examined whether ATP could competitively antagonize the effect of ZDV-3P. The results are shown in Table 1. Increasing concentrations of ATP induced an increase of both IC_{50} and n_H values of ZDV-3P.

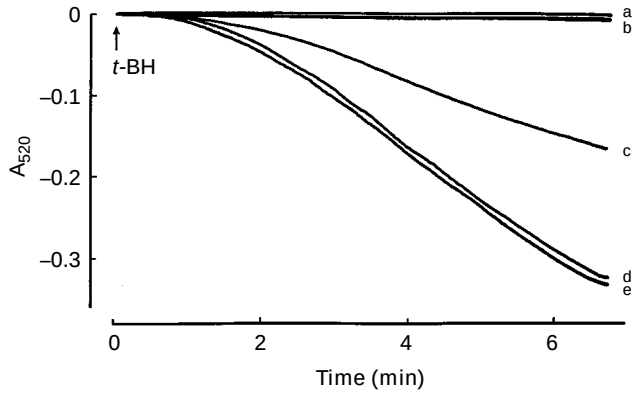

Figure 1 Comparison of the effect of ZDV-3P and ZDV on the permeability transition of isolated rat liver mitochondria. The permeability transition was monitored via mitochondrial swelling detected as a decrease in A_{520} with time. After 6 min of incubation of mitochondria without (control, line e) or with either 3 μM ZDV-3P or 100 μM ZDV, lines (c) and (d) respectively, 50 μM of Ca^{2+} were added. Four minutes later swelling was started by the introduction of 10 μM *tert*-butylhydroperoxide (*t*-BH). Lines (a) and (b): the same experimental procedure described for lines (c) and (d) was used except that Ca^{2+} and *t*-BH were not added.

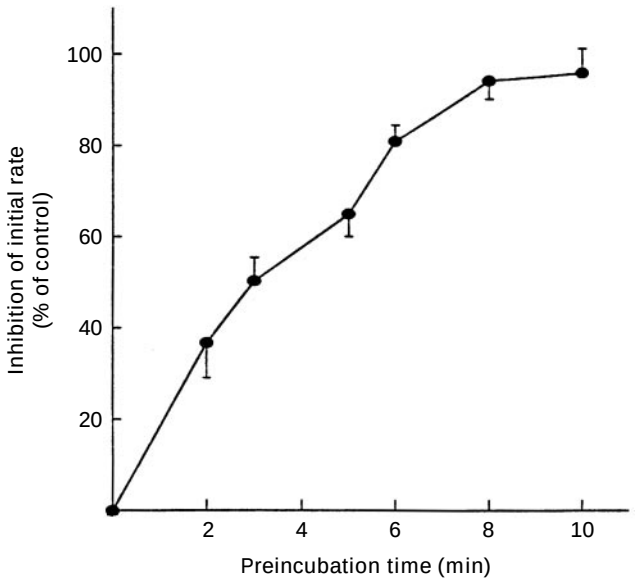

Figure 2 Influence of preincubation time with ZDV-3P upon its effect on rate of mitochondrial swelling. Mitochondria were preincubated at different times with a fixed concentration of ZDV-3P (8 μM) and swelling was initiated by the addition of Ca^{2+} (50 μM) and *tert*-butythydroperoxide (*t*-BH, 10 μM). The percentage inhibition of the initial rate of swelling after different incubation times with ZDV-3P was corrected for controls incubated for the same time without ZDV-3P. Results are expressed as means of three separate experiments; vertical lines show s.d. The initial swelling rate (control value) without preincubation time corresponds to 0.032 ± 0.004 absorbance units min^{-1} mg^{-1} protein.

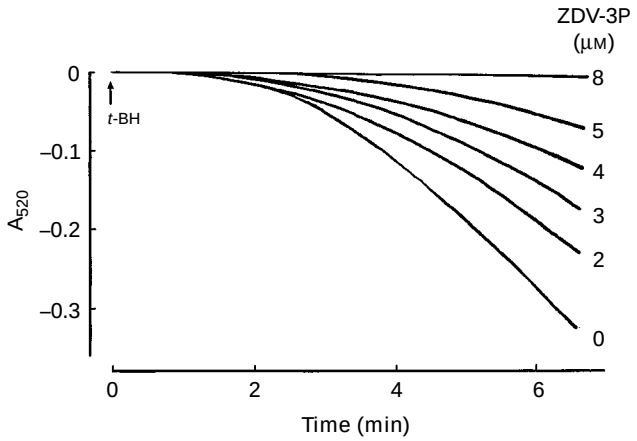

Figure 3 Concentration-dependence of the effect of ZDV-3P on the rate of mitochondrial swelling. After 6 min of incubation of mitochondria without (control) or with increasing concentrations of ZDV-3P, 50 μM of Ca^{2+} were added. Four minutes later, swelling was started by the addition of 10 μM *tert*-butylhydroperoxide (*t*-BH). Representative tracings from three separate experiments are shown.

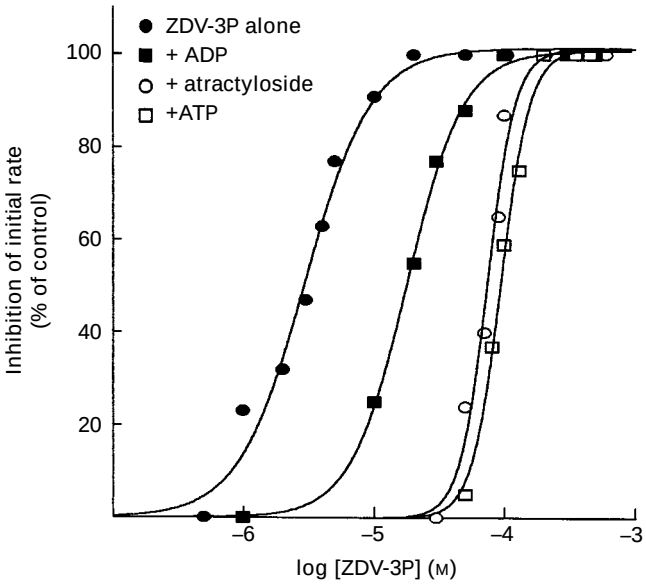

Figure 4 Effect of ADP, ATP or atracyloside upon the inhibitory effect of ZDV-3P on the rate of mitochondrial swelling. Mitochondria were incubated for 10 min with increasing concentrations of ZDV-3P alone or with ZDV-3P in the presence of 20 μM of either ADP, atractyloside or ATP. At t = 6 min, 50 μM Ca^{2+} were added and 4 min later the swelling was initiated by the addition of 10 μM *tert*-butythydroperoxide (*t*-BH). At this concentration (20 μM), ADP, ATP and atractyloside did not modify the maximal swelling. Data are expressed as % of the control value (maximal swelling), which in this particular experiment corresponded to 0.027 absorbance units min^{-1} mg^{-1} protein. Each inhibition curve is representative of three separate experiments.

Table 1 Effect of different concentrations of ATP upon the IC_{50} and n_H values of the inhibitory effect of ZDV-3P on mitochondrial swelling

ATP (μM)	*IC_{50}* (μM)	*n_H*
0	3.00±0.90	1.30±0.50
1	9.69±0.99**	1.44±0.23
5	20.25±1.49**	1.77±0.24
10	49.61±3.80**	1.83±0.25
20	66.67±6.45**	3.76±0.40*
70	165.9±1.98**	8.23±0.75**

Values are presented as means±s.d. of three separate experiments. *$P<0.03$, **$P<0.01$ versus the value obtained in the absence of ATP.

Table 2 Effect of different concentrations of ZDV upon the mitochondrial respiratory control and [ADP]/[Oxygen](P/O) ratios

ZDV (μM)	*RCR (as % of control)*	*P/O (as % of control)*
0 (control)	100	100
0.5	100	100
1	98±3	96±1
2	91±4	91±3
5	88±3*	84±2**

RCR (Respiratory control ratio) and P/O ([ADP]/[Oxygen]) values in the absence of ZDV (100%) were 4 and 1.6, respectively. Values are presented as means±s.d. of three separate experiments. *$P<0.05$, **$P<0.02$ versus the value obtained in the absence of ZDV.

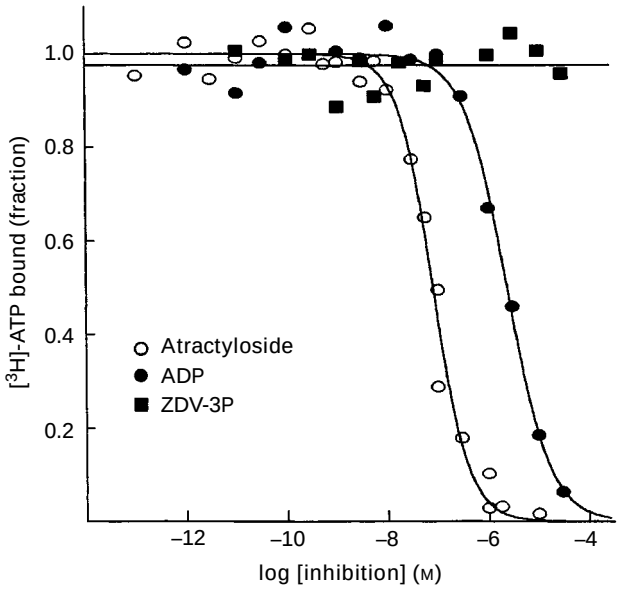

Figure 5 Effect of ADP, atractyloside and ZDV-3P on [^{3}H]-ATP binding to mitochondrial adenine nucleotide carrier (ANC). [^{3}H]-ATP (8 nM) was incubated with mitochondria (protein concentration = 0.1 mg^{-1} ml^{-1}) for 5 min at 25°C in the presence of increasing concentrations of either ADP, atractyloside or ZDV-3P. Abscissae: inhibitor concentrations. Ordinates show [^{3}H]-ATP binding as a fraction of the binding obtained in the absence of inhibitor.

Effect of ZDV-3P on [^{3}H]-ATP binding to mitochondria

The above results prompted us to investigate whether ZDV-3P could interact with the mitochondrial ANC. For this purpose, [^{3}H]-ATP was used as a marker of the mitochondrial ANC. Inhibition experiments showed that [^{3}H]-ATP binds to mitochondria and this binding was displaced by ATP, ADP and atractyloside. However, ZDV-3P up to a concentration of 100 μM did not displace [^{3}H]-ATP from its mitochondrial binding site(s) (Figure 5). The same results were obtained when mitochondria were subjected to hypoosmotic shock and sonication, a procedure which eliminated the [^{3}H]-ATP uptake component and, thus, reveals its binding component.

Comparison of the effects of ZDV and ZDV-3P on mitochondrial oxidative phosphorylation parameters

Table 2 shows that increasing the concentrations of ZDV decreased both the mitochondrial RCR and the P/O values. At 5 μM the decrease was 12% ($P<0.05$) and 16% ($P<0.02$), respectively. However, ZDV-3P up to a concentration of 10 μM did not affect these parameters. Whatever the concentration used, neither ZDV nor ZDV-3P affected the V_3.

Discussion

These data show that ZDV up to a concentration of 100 μM was unable to inhibit the MPT induced by *t*-BH in the presence of Ca^{2+}, whereas ZDV-3P inhibited this phenomenon with an IC_{50} in the micromolar range. To our knowledge this is the first study which describes such an inhibitory effect of ZDV-3P. Interestingly the drug concentrations used in this study were in the range of the therapeutic ones. ZDV-3P must be preincubated with mitochondria in order to exert its inhibitory effect and this incubation period probably reflects the kinetics of ZDV-3P binding or its uptake by mitochondria.

The inhibitory effect of ZDV-3P was antagonized by ATP, ADP and atractyloside. These three compounds have already been documented as MPT inducers of non-energized mitochondria (Halestrap & Davidson, 1990). Our data support these previous findings. However, opposite effects, i.e. MPT inhibition, have also been observed with ADP and ATP on energized mitochondria (Carbonera & Azzone, 1988; Marty *et al*., 1992). We have no clear explanation for these differences. A possible hypothesis could be that under non-energized conditions a conformational change of the ATP and ADP sites might occur, leading to MPT enhancement.

The fact that ZDV-3P competes with ATP, ADP and atractyloside, which are ANC specific ligands, supports previous findings showing that the ANC is involved in MPT (Carbonera & Azzone, 1988; De Macedo *et al*., 1993). It would suggest that ZDV-3P exerts its inhibitory effect by interacting with the ANC. Although ZDV-3P and ATP are nucleoside analogues, our data show that their binding sites are distinct but not independent as increasing concentrations of ATP increased the cooperativity of ZDV-3P, suggesting that ZDV-3P binding site(s) is (are) influenced by ATP.

Another mechanism by which ZDV-3P could inhibit the MPT is through inhibition of mitochondrial Ca^{2+} influx preventing its interaction with an internal divalent cation site, which when occupied by Ca^{2+}, increases the probability of the open state (Szabo *et al*., 1992; Bernardi *et al*., 1993). We observed that ZDV-3P did not inhibit either mitochondrial influx or mitochondrial efflux of Ca^{2+} (results not shown). Similarly ZDV-3P affected neither the mitochondrial RCR, nor the P/O ratio, a parameter which indicates mitochondrial ATP synthesis efficiency, whereas the prodrug ZDV did inhibit the above parameters in a concentration-dependent manner. Taken together, these results strongly indicate that both ZDV and ZDV-3P interact with mitochondrial functions but in different ways.

The finding that ZDV-3P at high concentrations did not inhibit the mitochondrial respiration at state 3 (ADP stimulated respiration) suggests that this drug is unable to inhibit the

ADP/ATP exchange which weakens the hypothesis of a direct interaction of ZDV-3P with the ANC. In order to verify this finding, [^{3}H]-ATP was used as a marker of the mitochondrial ANC. The [^{3}H]-ATP interaction with intact mitochondria is considered to represent the combination of two phenomena, [^{3}H]-ATP binding and its uptake, our results showed that ADP, ATP and atractyloside, the three known specific ligands of the mitochondrial ANC, inhibited both of them, whereas ZDV-3P did not. The same results were found when mitochondria were subjected to hypoosmotic shock and subsequent sonication, a procedure with eliminates any possible [^{3}H]-ATP uptake and reveals its binding. This confirmed that ZDV-3P neither displaces [^{3}H]-ATP from its binding site(s) nor prevents its uptake by mitochondria. These results are in accordance with the absence of any effect of ZDV-3P on mitochondrial respiration rate at state 3. Taken together, these findings show that ZDV-3P does not interact with the mitochondrial ANC. Further experiments are required in order to locate the ZDV-3P mitochondrial binding site(s).

Strong evidence has already accumulated that mitochondria from muscular cells may also be a cellular target of ZDV. Indeed, Lamperth *et al*. (1991) have found a decreased RCR by examining the effect of ZDV *in vitro* on human muscle in tissue culture. In *in vivo* studies on animals, Lewis *et al*. (1992) have shown the onset of cardiomyopathy in rats fed with ZDV, accompanied by disorganization and disappearance of mitochondrial cristae. Sinnwell *et al*. (1995), by using *in vivo* ^{31}P magnetic resonance spectroscopy in order to follow phosphorylated metabolites during exercise in patients on long-term ZDV therapy, have found that this treatment decreased the maximal work output and the maximal rate of muscle ATP synthesis.

All these data suggest that ZDV treatment may induce both a RCR decrease and an alteration in ATP synthesis. They are in agreement with our own data obtained with hepatocytes. Moreover, Chen *et al*. (1992) have found that ZDV concomitantly induced hepatotoxicity and myopathy in patients treated for HIV infection. This is not surprising as mitochondria of both types of cells have fundamentally the same ATP synthesis mechanisms differing only in the respective rates of their respiratory complexes and carriers, adapted to different demands of energy (Korzeniewski & Marzat, 1996). So, it is reasonable to assume that mitochondrial ATP generation mechanisms do not differ between the cells and are not impaired by ZDV-3P.

It is also possible that the MPT generation, leading to the giant mitochondria observed during ZDV chronic administration, may be a secondary result of a cellular toxic effect of the drug, for instance at the level of the nucleus (Sommadossi *et al*., 1989). However, we cannot confirm this at present since our work was performed on isolated mitochondria.

In conclusion, the effects of ZDV-3P observed *in vitro* on rat hepatocytes ruled out any involvement of ZDV-3P in the mitochondrial alterations observed in man during ZDV administration. This is in accordance with the results recently presented by Sales *et al*. (1996), who have shown that the toxic effects of ZDV-3P observed on several lymphoblastoid cell lines were not associated with ZDV-3P concentration. The most interesting finding of this work is that the active form of ZDV (ZDV-3P) in the body is not the compound which induces mitochondrial toxicity.

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