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REVIEW

Mitochondria as Cell Targets of AZT (Zidovudine)

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ABSTRACT. 1. The subject of this review is the interaction between AZT (zidovudine) and mitochondria as described in papers dealing with AZT therapy both in AIDS patients and in model systems—that is, in cultured cells and in isolated mitochondria.

2. The structure and function of mitochondria are briefly described with discussion of the theoretical frame for a detailed bioenergetic investigation.

3. Experimental work is reported showing that mitochondria are cell AZT targets: changes in the structure and function induced by long-term AZT therapy as investigated both in AIDS patients and in model systems.

4. The AZT inhibition of energy-supplying reactions is considered in detail in studies dealing with long-term treatment and studies in which AZT was added to isolated mitochondria. In particular, adenylate kinase, ADP/ATP translocase and DNA polymerase γ are reported as molecular targets of AZT.

5. Some perspectives of AZT therapy from the study of the effect of AZT on mitochondrion biochemistry are briefly reported. GEN PHARMAC 31;4:531–538, 1998. **© 1998 Elsevier Science Inc.**

KEY WORDS. Mitochondria, 3′-azido-3′-deoxythymidine (AZT), acquired immuno-deficiency syndrome, AZT toxicity

INTRODUCTION

3′-Azido-3′-deoxythymidine (AZT, zidovudine) is the first drug, in use since 1986, in the therapy of acquired immunodeficiency syndrome (AIDS), because it is the most effective compound of a class of nucleoside analogues that can inhibit the replication of the human immunodeficiency virus 1 (HIV-1) (Fischl *et al.*, 1987; Mitsuya and Broder, 1987; Yarchoan *et al.*, 1986). The antiretroviral activity of AZT proved to derive from its conversion into AZT triphosphate (AZTTP) catalyzed by the enzymes of the thymidine-phosphorylation pathway—that is, thymidine kinase (EC 2.7.1.21), thymidylate kinase (EC 2.7.4.9) and the nucleoside diphosphate kinase (EC 2.7.4.6) (Balzarini *et al.*, 1989; Furman *et al.*, 1986). AZTTP was found both to inhibit HIV-1 reverse transcriptase and to terminate the newly synthesized viral DNA chain (Hao *et al.*, 1988).

Unfortunately, in AIDS therapy, the clinical efficacy of AZT is limited by its toxic side effects, which are principally directed to the bone marrow and skeletal cardiac muscle. As a result of long-term treatment of patients with AZT, anemia, leukopenia (Richman *et al.*, 1987), myalgia, muscle weakness and elevated serum creatine kinase levels (Dalakas *et al.*, 1990) were observed, which required the discontinuation of the therapy.

To prevent AZT side effects, the elucidation of the processes leading to AZT cytoxicity is needed. Different hypotheses have been suggested to account for the biochemical mechanism(s) responsible for the cytotoxic effects induced by AZT in human host cells: namely, the decrease in physiological levels of thymidine triphosphate (TTP) and other deoxyribonucleotides needed for host-cell DNA synthesis (Frick *et al.*, 1988; Mitsuya and Broder, 1987); AZT incorporation into newly synthesized host-cell DNA (Sommadossi *et al.*, 1989), with inhibition of the elongation by chain termination (Copeland *et al.*, 1992); the formation of 3′-amino-3′-deoxythymidine, a highly toxic catabolite (Cretton *et al.*, 1991); and the inhibition of protein glycosylation and nucleotide–sugar import into the Golgi complex (Hall *et al.*, 1994).

However, both because energy-related pathologies (Dalakas *et al.*, 1989) were observed in the AZT-treated patients and in the light of the pioneering *in vitro* studies by Simpson *et al.* (1989), the possibility that mitochondria were the cell targets of the toxic effects of AZT was considered. Nevertheless, the mechanisms by which AZT–mitochondrion interaction takes place remain as yet far from being exhaustively elucidated.

In this review, we report studies that describe the effect of AZT on mitochondrial structure and function with special attention paid to molecular studies dealing with the interaction between AZT and mitochondrial components taking part in oxidative phosphorylation.

MITOCHONDRIA AS THE CELL ENERGY SOURCE

For a better understanding of the AZT–mitochondrion interaction, a survey of mitochondrial features is presented here. Mitochondria are the cellular sites of the majority of energy-supplying biochemical reactions. A mitochondrion consists of four compartments that differ from one another both in chemical composition and in enzyme profile: the smooth outer membrane, the folded inner membrane, the intermembrane space and the inner membrane delimited ma-

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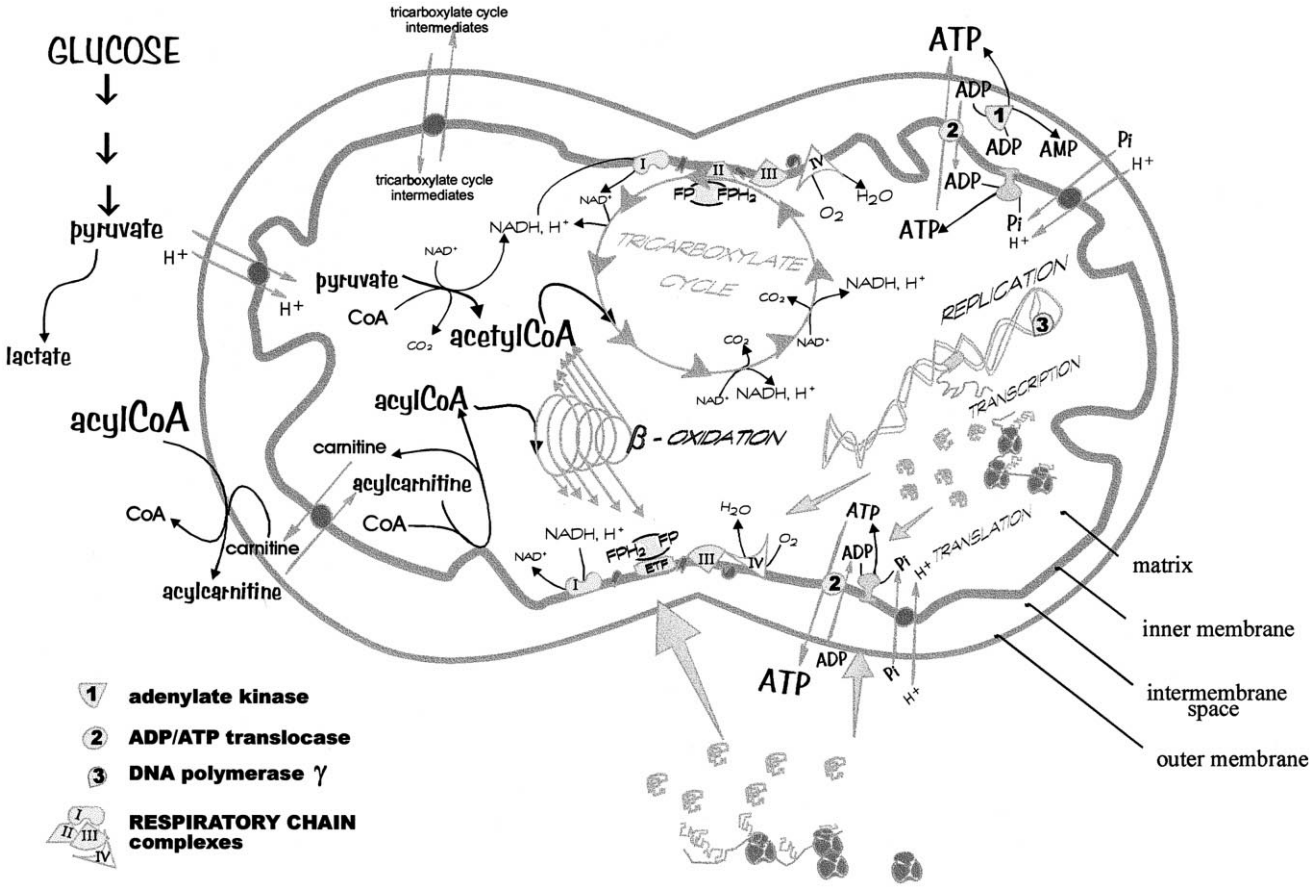

FIGURE 1. Mammalian mitochondrion. The reported AZT targets are numbered.

trix. Each compartment plays a specific role in mitochondrial metabolism and, consequently, in the functional integrity of the whole cell (Fig. 1).

In the matrix, respiratory substrates are oxidized by their specific dehydrogenases, mainly through the tricarboxylate cycle and the β-oxidation pathway, thus generating NADH and reduced flavoproteins. Reducing equivalents are transferred to molecular oxygen through the respiratory chain, which consists of four respiratory complexes and two mobile components, ubiquinone and cytochrome *c*. The electron flow generates the transmembrane electrochemical proton gradient ($\Delta\mu_H^+$), which is, according to Mitchell's chemiosmotic theory, the coupling vehicle between substrate oxidation and ADP phosphorylation to ATP, which is catalyzed by the F_o–F_1–ATP synthase.

To become available in the cytosol, ATP is transported outside mitochondria in exchange for incoming ADP by the ADP/ATP translocase. Consistently, the traffic of phosphate and other metabolites from the cytosol to the matrix enzymes and vice versa is mediated by many specific translocases, located in the inner membrane.

Thus, it should be noted that the impairment of oxidative phosphorylation can be a result of the impairment of one or more of the reported steps, each of them being dependent on several reactions. To ascertain the molecular mechanism by which different molecules—among them, for instance, AZT—can affect the mitochondrial function, a detailed analysis is required. In particular, the *in vitro* oxygen uptake and ATP synthesis have to be investigated. The theoretical framework of such an investigation is briefly reported here: oxidative phosphorylation, as simply investigated *in vitro* with coupled mitochondria, externally added respiratory substrates and ADP, derives from several steps: metabolite transport across the inner mitochondria membrane mostly by *specific translocators;* intramitochondrial substrate oxidation catalyzed by specific *dehydrogenase;* electron flow along the *respiratory chain;* $\Delta\mu_H^+$ *generation;* ADP uptake into mitochondria by *ADP/ATP translocase;* and $\Delta\mu_H^+$ utilization with ATP synthesis catalysed by F_o–F_1–*ATP synthase*. Finally, it should be noted that mitochondrial reactions, using the newly synthesized ATP, can regulate the cell energy charge and the adenine nucleotide pool—for instance, by *adenylate kinase*.

Moreover, in this, as in other similar multistep processes, to identify the real molecular target(s), it is crucial to define the rate-limiting step; in fact, in some cases, it might not be possible to reveal the inhibition of one of the faster steps. Because the rate of the enzymatic reaction depends on a number of factors including the nature and the amount or activity of the enzyme(s), the nature and the concentration of substrate and the cofactors, pH profile and the nature and the concentration of effectors, the elucidation of the molecular mechanism by which certain alterations derive remains a very hard task. Another point to be considered is in regard to the *in vitro/in vivo* situation: this point raises the question of whether the reported *in vitro* effects can occur *in vivo*. In this case, the effector concentration must also be considered, thus excluding all the experimental findings in which the drug used *in vitro* exceeds the pharmacological dose.

Mitochondria can themselves contribute to their biogenesis; in fact, they possess some copies of circular naked DNA and the replication, transcription and translation enzymatic "kits" that allow for

the duplication of mitochondrial DNA (mtDNA) and the synthesis of the 2 rRNAs and the 22 tRNAs. Thirteen hydrophobic polipeptides, which are subunits of respiratory chain complexes and ATP synthase, are synthesized within the organelle. The remaining mitochondrial proteins are coded by the nuclear genome, translated on cytosol polysomes and imported into mitochondria in accord with a pattern that requires proteinaceous complexes located at the contact sites between the two membranes. Thus, during the cell cycle, a precise coordination between the two genomes is required to allow for the growth and renewal of mitochondrial components.

MITOCHONDRIA AS CELL AZT TARGETS

The history of the identification of the mitochondrion as a cellular target of AZT began in 1989 on the basis of both clinical observations made on HIV-infected patients (Dalakas *et al.*, 1989, 1990) and measurements carried out on isolated mitochondria *in vitro* (Simpson *et al.*, 1989). Dalakas proposed that long-term treatment with AZT induces a toxic mitochondrial myopathy characterized by various changes in the mitochondrial structure, which are independent of HIV infection. On the other hand, Simpson found that AZT, externally added to isolated mitochondria, can inhibit the replication of mitochondrial DNA. In both cases long-term effects were under investigation in AIDS patients and in model systems. The papers concerning these two topics will be discussed separately.

Changes in the structure and function of mitochondria induced by long-term AZT therapy in AIDS patients

The clinical investigations of mitochondrial toxicity induced by long-term AZT therapy are summarized in Table 1. In these studies, the main experimental models were biopsy specimens taken from skeletal and heart muscle of HIV-infected patients. Several experimental findings have shown that a toxic mitochondrial myopathy arises as a result of the treatment with AZT (1.0–1.2 g drug/day, for 2–48 months). Such a myopathy is characterized by red ragged fibers and various mitochondrial abnormalities including enlarged size, abnormal cristae and abnormal proliferation of the organelle, as observed by both light and electron microscopy (Chen *et al.*, 1992; Cupler *et al.*, 1995; Pezeshkpour *et al.*, 1991).

Furthermore, long-term AZT therapy induces a severe mtDNA depletion in skeletal muscle, as demonstrated by the measurements of mtDNA content (Arnaudo *et al.*, 1991; Casademont *et al.*, 1996); however, Southern blot analysis revealed no abnormality in mtDNA, thus ruling out both duplication and large deletion of mtDNA (Mhiri *et al.*, 1991).

An increase in both serum lactate concentration and creatine kinase activity was found in AIDS patients treated for 12–48 months with AZT, further suggesting a mitochondrial dysfunction balanced by an increase in anaerobic glycolysis and in creatine-dependent ATP synthesis (Aggarwal *et al.*, 1996; Mhiri *et al.*, 1991; Peters *et al.*, 1993; Simpson *et al.*, 1993); in addition, changes in phosphocreatine, ATP and intracellular pH were observed in skeletal muscle during a graded physical exercise of patients receiving AZT (Sinnwell *et al.*, 1995).

A considerable reduction was also found in the total muscle amount of carnitine, which allows for the translocation inside mitochondria of acyl CoA in the route to β-oxidation (Dalakas *et al.*, 1994). As a result of the decrease in the β-oxidation rate, an accumulation of lipid droplets within the muscle fibers also was observed (Cupler *et al.*, 1995; Dalakas *et al.*, 1994; Mhiri *et al.*, 1991; Tomerelli *et al.*, 1992).

Although the majority of the reported clinical investigations consider the skeletal and cardiac muscle the preferential tissues specifically affected by the toxic effects of AZT, some mitochondrial alterations have also been observed in other tissues of AZT-treated patients, such as the liver (Chen *et al.*, 1992; Olano *et al.*, 1995).

In all the preceding investigations, no significant attempt was made to elucidate the molecular mechanism responsible for the reported toxic effects.

Changes in the structure and function of mitochondria induced by long-term AZT therapy as investigated in model systems

In the light of mitochondrial changes induced by long-term AZT treatment, Lamperth *et al.* (1991) used experimental "model systems," in both *in vivo* studies, carried out with muscle biopsy taken from healthy AZT-treated rats, and *in vitro* studies, carried out with tissue cultures. Because rats are not infected by HIV, AZT-treated rats are a good model for the study of AZT-induced myopathy.

In rats, a preferential accumulation of the drug in skeletal muscle and heart was observed and, in these tissues, enlarged mitochondria with disorganized or absent cristae and electron-dense deposits in their matrix were observed. As a consequence of mitochondrial dysfunction, creatine kinase activity, both serum lactate and glucose increase, was measured. Myotubes in tissue culture consistently exhibited abnormal mitochondria characterized by proliferation, enlarged size, abnormal cristae and electron-dense deposits in their matrix.

The capability of AZT to induce ultrastructural changes and biochemical alterations of mitochondria was further demonstrated and described in some detail in other studies (Table 2). Myoblasts and myotubes were the cells most frequently used, even though consistent results have been obtained for murine erythroleukemia cells (Friend cells), human spinal ganglia and spinal cordon cells, fibroblasts from patients affected by a mitochondrial disease and human lymphocytes. As a result of these studies, a strong inhibition of cell replication induced by AZT was evidenced, accompanied by a severe reduction of both mtDNA and mtRNA content and decreased mitochondrial polypeptide synthesis.

Consistently, an increase in lactate concentration (Hobbs *et al.*, 1995) and a considerable accumulation of lipid droplets (Aggarwal *et al.*, 1997; Corcuera *et al.*, 1996; Hobbs *et al.*, 1995; Semino-Mora *et al.*, 1994a) within the cells were observed both in cell cultures and in rats.

In spite of the model system used, to observe deleterious effects on mitochondria, long-lasting and high doses of externally added AZT are required [3 weeks and 3 months are the minimum times to observe these effects in muscle tissue culture and in rats, respectively, in Lamperth *et al.*, (1991)].

THE MECHANISM(S) OF AZT-INDUCED MITOCHONDRIAL ALTERATIONS

In spite of the demonstration of a number of structural and functional mitochondrial modifications, owing to long-term AZT administration to AIDS patients, rats and isolated cells, the answer to the question of what the molecular mechanism(s) responsible for these effects is remained unsolved and was considered in a further series of investigations carried out at the molecular level.

Inhibition of mtDNA replication induced by long-term treatment with AZT

mtDNA replication was the first mitochondrial pathway considered to explain the toxic effect of AZT. Simpson *et al.* (1989) demon-

TABLE 1. Experimental model: patients

Author	Year	Source	Observed effect
Dalakas *et al.*	1990	Skeletal muscle	Mitochondrial structural changes; depletion of mtDNA
Arnaudo *et al.*	1991	Skeletal muscle	Depletion of mtDNA (78%)
Pezeshkpour *et al.*	1991	Skeletal muscle	Ragged red fibers; abnormal mitochondria with paracrystalline inclusions; protein–lipid complexes within the mitochondrial matrix
Mhiri *et al.*	1991	Skeletal muscle	Reduced activity of some respiratory chain enzymes; high levels of serum CK, glycogen excess and accumulation of lipid droplets; no change in mtDNA content
Chariot and Gherardi	1991	Skeletal muscle	Partial COX deficiency
Chen *et al.*	1992	Skeletal muscle and liver	Enlarged mitochondria with paracrystalline inclusions
Tomerelli *et al.*	1992	Skeletal muscle	Changes in number, size and structure of mitochondria; repleted fibers with lipid droplets; partial COX and Cyt *c* reductase deficiency
Weissman *et al.*	1992	Calf muscles during physical exercise	Reduced recovery of phosphocreatine during physical exercise with significant delaying of the mitochondrial oxidative function
Peters *et al.*	1993	Skeletal muscle	Lipid accumulation and grossly giant mitochondria; increase in serum CK levels
Simpson *et al.*	1993	Skeletal muscle	Structural abnormalities of mitochondria; high serum CK levels
Dalakas *et al.*	1994	Skeletal muscle	Lipid accumulation; reduction of carnitine levels
Sinnwell *et al.*	1995	Gastrocnemius muscle	Changes in phosphocreatine, ATP and intracellular pH during a graded steady-state physical exercise
Olano *et al.*	1995	Skeletal muscle and liver	Massive hepatomegaly and steatosis; enlarged mitochondria in the liver; no mitochondrial changes in the skeletal muscle
Morgello *et al.*	1995	Skeletal muscle	Abnormal mitochondria
Cupler *et al.*	1995	Skeletal muscle	Proliferation of normal and abnormal mitochondria and increased amounts of lipid, glycogen and lipofuscin
Aggarwal *et al.*	1996	Skeletal muscle	Accumulation of lactate with consequent severe metabolic acidosis
Casademont *et al.*	1996	Skeletal muscle	Depletion of mtDNA

Abbreviations: CK, creatine kinase; COX, cytochrome *c* oxidase.
Mode of administration: pharmacological therapy with AZT (1.0–1.2 g/day). Time of drug administration: 2–39 months (mean 20).

strated the inhibition in mtDNA replication caused by AZT addition to mitochondria isolated *in vitro:* AZT proved to be a strong inhibitor of the incorporation into mtDNA of both [^{3}H]thymidine and [^{3}H]dATP. In this work, Simpson proposed that the observed drastic reduction of mitochondrial DNA content was dependent on the inhibition of DNA polymerase γ, the matrix enzyme taking part in mitochondrial DNA synthesis, by AZT. In the past few years, a wide consensus on such a proposal has been reported, thus making DNA polymerase γ the "primary" mitochondrial target responsible for the AZT-induced alteration of the replication process and, consequently, for mitochondrial damage.

The AZT-dependent inhibition of mitochondrial DNA polymerase γ activity has been confirmed and investigated in detail with kinetic studies carried out by using AZTTP with enzymes isolated from different sources (Table 3). AZTTP has been shown to be a powerful competitive–mixed competitive inhibitor of the natural substrates (K_i<1 μM), whereas AZTTP at higher concentration can work as a chain terminator of γ DNA polymerase (Table 3). According to the points raised in the section on mitochondria as the cell energy source, one could argue that *in vitro* studies carried out with isolated enzymes could not mirror the *in vivo* situation. In fact, the inhibition degree obviously depends on the inhibitor K_i and on the steady-state concentration of both the substrate and the inhibitor. Furthermore, as far as AZT therapy is concerned, the AZTTP concentration in the mitochondria of patients after long-term therapy is unknown. In particular, the sequence of events occurring *in vivo*, starting from AZT uptake by the cell [which seems to occur by a nonfacilitated mechanism described by Zimmerman *et al.* (1987)] and leading to internalization in the mitochondrial matrix space, is far from being completely understood. An alternative proposal to explain the biochemical origin of mtDNA alteration suggests that it is a result of a phenotypic expression of mutant mtDNA caused by oxygen radicals, which are responsible for a massive conversion of guanosine into 8-OH-guanosine observed in mouse liver mtDNA after AZT administration (Hayakawa *et al.*, 1991).

AZT as an inhibitor of energy-supplying reactions

That mitochondrial bioenergetics are actually impaired by long-term therapy with the drug, in terms of either ATP deficiency syndrome (Simpson *et al.*, 1989) or energy shortage [in Till's terms (Till and McDonell, 1990)], was demonstrated by using two kinds of experiments. In one case, modification of the activity in certain partial steps of tricarboxylate cycle/electron flow owing to externally added AZT/AZT derivative was investigated. In the second case, use was made of isolated, coupled mitochondria, with investigation of the main reactions leading to the oxidative phosphorylation.

The first approach was developed both in "model systems" and with patients and essentially consists of measuring enzymatic activities after long-term administration of AZT. No significant activity modification of citrate synthase, a regulatory enzyme of the Krebs cycle, was found after long-term AZT therapy, as measured both in patient muscle homogenate (Mhiri *et al.*, 1991) and in human muscle cells (Herzberg *et al.*, 1992). Consistently, the activities of other Krebs cycle enzymes, including isocitrate, succinate, and malate dehydrogenase, as histochemically revealed in rat cardiac muscle

TABLE 2. Studies carried out with model systems

Author	Year	Experimental model	Observed effect
MODEL SYSTEM: RATS			
Lewis *et al.*	1991	Cardiac muscle	Swelling and cristae disruption; mtRNA encoding for Cyt *b* subunit reduction
Lewis *et al.*	1992	Striated skeletal muscle	Swelling and cristae disruption, myelin figures; decreased mtDNA, mtRNA and mitochondrial polypeptide synthesis
Corcuera *et al.*	1994	Cardiac muscle	Increased size of mitochondria; cristae disruption; no changes in isocitrate, succinate, malate, NADH and NADPH dehydrogenase activities
Linnane *et al.*	1995	Skeletal and cardiac muscle	Myopathy with decrease in the steady-state level of soleus force performance and in the activity of complex I; improvement due to ubiquinone administration
Corcuera *et al.*	1996	Liver	Histological alterations—i.e., mitochondrial swelling; ultrastructural alterations—i.e., glycogen and lipid accumulation
MODEL SYSTEM: CELL CULTURES			
Hobbs *et al.*	1992	Friend cells	Strong inhibition of cell replication; depressed mtDNA replication, no effect on hemoglobin synthesis
Herzberg *et al.*	1992	Human muscle cultures	Strong inhibition of cell replication; no effect on citrate synthase and COX activities
Semino-Mora *et al.*	1994a	Human myotubes	Swelling and cristae disruption; lipid droplet accumulation; L-carnitine, used with AZT, prevents the structural alteration of mitochondria and the accumulation of lipids
Semino-Mora *et al.*	1994b	Human myotubes	Abnormal mitochondria with paracristalline inclusions; treatment with L-carnitine improves and preserves the AZT-induced changes
Hobbs *et al.*	1995	Friend cells	Marked inhibition of cell replication and ATP synthesis in mitochondria from cells incubated for 5 days with AZT (long-term effect); inhibition of cell replication and increase in mitochondrial proliferation within 3 hr after addition of AZT, changes in ATP and lactate levels (short-term effect)
Schröder *et al.*	1996	Cocultures of human spinal ganglia, spinal cord and skeletal muscle from fetal rats	Swelling, loss of cristae
Wang *et al.*	1996	Wild-type and cultured Kearns-Sayre syndrome fibroblasts	Depletion of wild-type mtDNA levels and increase in deleted mtDNA levels
Agarwal and Olivero	1997	Human lymphocytes H9 culture cells	Mitochondrial damage; elevated accumulation of neutral intracellular lipid deposits

(Corcuera *et al.*, 1994), were found to be unaffected by long-term AZT administration.

As far as the respiratory chain enzymes are concerned, the following results have been reported: no impairment of cytochrome *c* oxidase (COX) activity was found in human muscle cell cultures (Herzberg *et al.*, 1992); on the other hand, a partial reduction of the activity of certain respiratory chain complexes was observed in muscle from AZT-treated AIDS patients, the enzymes that exhibit the maximum percentage of inhibition being succinate-cytochrome *c* reductase and, mostly, COX (Mhiri *et al.*, 1991; Tomerelli *et al.*, 1992). The induction of a decline in mitochondrial respiratory function by AZT has been demonstrated by using electron transfer phosphorylating submitochondrial particles isolated from long-term treated rat hearts. Measurements were made of the membrane potential generated by either NADH or succinate oxidation. As a conclusion of these studies, complex I was suggested to be the most affected respiratory complex. Interestingly enough, this damage was found to be restored by ubiquinone administration (Linnane *et al.*, 1995).

In our opinion such an investigation could not provide useful information concerning the possibility that inhibition found *in vitro* could also be significant *in vivo*. This information, in fact, requires knowledge of the degree of control exerted by each single step on electron flow (see the section on mitochondria as the cell energy source). An alteration in respiratory chain activity has also been demonstrated by studies in which the respiratory capacity of mitochondria isolated from long-term AZT-administered rats was polarographically tested (Lamperth *et al.*, 1991). A tissue specificity in the AZT-induced effect was found. In skeletal muscle, but not in brain mitochondria, a significant decrease in succinate-cytochrome *c* reductase and rotenone-sensitive NADH cytochrome *c* reductase (complexes I+III) activities was found, as assessed by measurements carried out in state 3 respiration. Moreover, in brain, the glutamate oxidation rate, as measured in state 4 respiration, is threefold higher than the control, so a significant decrease in the respiratory control ratio was measured (1.9 versus 6.0 in the control), which means that long-term administration of AZT could uncouple mitochondrial respiration from oxidative phosphorylation.

TABLE 3. Inhibition of DNA polymerase γ by AZTTP

Author	Year	Source of enzyme	K_m (for dTTP) (μM)	K_i (for AZTTP) (μM)	AZTTP action mechanism
Ono *et al.*	1989	KBIII human cells	0.63	0.1	Competitive DNA polymerase γ inhibitor
Izuda *et al.*	1991	Bovine calf thymus	2	26	Competitive DNA polymerase γ inhibitor chain terminator (<200 μM)
Lewis *et al.*	1994	Bovine heart	0.8	1.8 (K_i) 6.8 (K_i')	Mixed competitive DNA polymerase γ inhibitor
Eriksson *et al.*	1995	Recombinant yeast	0.01	*nd*	Chain terminator

In spite of the foregoing consideration, the inhibition of electron flow that takes place after long-term treatment with AZT could be due to a decrease in the activity of the complexes investigated owing to the inhibition of mtDNA replication (Simpson *et al.*, 1989). In fact, in humans, certain subunits of respiratory-chain complexes are coded by mtDNA.

Short-term effects on mitochondria induced by AZT

More recently, investigation has been carried out on mitochondria isolated *in vitro* aimed at ascertaining whether and how AZT itself could impair energy metabolism. Even though such work appears to be simpler than the aforedescribed approach, a detailed investigation, carried out as outlined in the section on mitochondria as the cell energy source, remains necessary. Thus, for instance, in the light of the aforeraised points, we consider it difficult to accept, as a possible explanation of the toxic effect of AZT, the inhibition of electron transfer through respiratory enzyme complex I and the induced tissue-specific inhibition of succinate-linked respiration in intact mitochondria isolated from rat skeletal muscle reported by Modica-Napolitano (1993). In this paper, the AZT concentrations used were from two or three orders of magnitude higher than pharmacological plasma levels, so, in spite of its occurrence *in vitro,* such an inhibition is expected not to occur *in vivo.* The effect of AZT on the activity of adenylate kinase, which regulates the adenine nucleotide pool, has been investigated (Barile *et al.*, 1994). In this case, up to 15 μM AZT was used, thus mimicking the *in vivo* situation. Binding of AZT to the enzyme was fluorimetrically shown; AZT was found to strongly inhibit adenylate kinase in the direction of ATP synthesis (K_i, 8 μM). Experiments on isolated intact rat liver mitochondria with the enzyme activity measured in both directions confirmed the isolated enzyme results. The respiratory control index was found not to be affected by AZT, thus suggesting that AZT does not uncouple oxidative phosphorylation.

Clear evidence that AZT can itself enter mitochondria was shown by Barile *et al.* (1997), measuring [^{14}C]AZT uptake by rat liver mitochondria *in vitro.* AZT accumulated in mitochondria in a time-dependent manner. The rate of AZT uptake into mitochondria showed a hyperbolic dependence on the drug concentration and was inhibited by mersalyl, a thiol reagent that cannot enter mitochondria, thus showing that a membrane protein takes part in AZT transport. Investigation into the capability of AZT to affect certain mitochondrial translocases demonstrated that AZT had no effect on P_i, dicarboxylate, tricarboxylate or oxodicarboxylate translocators. In contrast, AZT was found to inhibit ADP/ATP antiport in either mitochondria or mitoplasts in a competitive manner with different sensitivities. The failure both of carboxyatractyloside to inhibit AZT transport into mitochondria and of AZT to cause ATP efflux from ATP-loaded mitochondria showed that AZT does not use the ADP/ATP translocator to enter mitochondria.

These papers, showing two mitochondrial AZT targets (adenylate kinase and ADP/ATP translocator), make it possible that their impairment by AZT is among the biochemical causes for the ATP-deficiency syndrome. This conclusion could also explain the results of other studies on the growth and metabolism of Friend cells. In fact, subsequent to cell incubation with 5 μM AZT, changes in lactate, ATP synthesis and O_2 uptake were measured. Some of these effects occurred as early as 3 hr, thus excluding the possible implication of DNA polymerase γ (Hobbs *et al.*, 1995). The failure of AZT in uncoupling mitochondria was further suggested in the same paper; unfortunately the experimental data shown to assess this point are not well defined, thus making it difficult to accept the conclusion. However, in another work, carried out by comparing the effect of AZT and the uncoupler FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) on several parameters including the rate of oxygen consumption, the mitochondrial NAD(P)H oxidation, the stimulation of ATP hydrolysis, mitochondrial swelling, and so forth, it was concluded that AZT is not an uncoupler (Atlante and Passarella, 1998).

PERSPECTIVES IN AZT THERAPY

In the light of the reported findings, we conclude that the early primary targets of AZT are the mitochondrial adenylate kinase and ADP/ATP translocator. Because AZT accumulation in the intermembrane space is possible (Barile *et al.*, 1997), we think that inhibition both of adenylate kinase and of the ADP/ATP translocase could take place *in vivo,* with a consequent decrease in the cell ATP availability. To prevent this damage, the development of AZT derivatives that can exert no effect on the mitochondrial enzyme/translocase while maintaining their therapeutic effect is worthwhile.

Because AZT and perhaps AZTTP can enter mitochondria, we consider the *in vivo* inhibition of DNA polymerase γ with a long-term impairment of the electron flow through the respiratory chain a probable result of their uptake, even though AZTTP formation inside the matrix cannot be excluded. Thus, we feel that the identification of the mitochondrial translocase that translocates AZT/AZTTP into mitochondria should be a primary goal, thus making a therapeutic treatment possible in which AZT uptake by mitochondria is prevented. In this case, the physiological substrate might be added together with AZT/AZTTP in the hope that this could compete with AZT, thus preventing its uptake.

The mitochondrial AZT targets reported to date are indicated in Figure 1.

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