

Mitochondria as the target for mildronate's protective effects in azidothymidine (AZT)-induced toxicity of isolated rat liver mitochondria

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Previously mildronate, an aza-butYRObetaine derivative, was shown to be a cytoprotective drug, through its mechanism of action of inhibition of carnitine palmitoyltransferase-1, thus protecting mitochondria from long-chain fatty acid accumulation and subsequent damage. Recently in an azidothymidine (AZT)-induced cardiotoxicity model *in vivo* (in mice), we have found mildronate's ability of protecting heart tissue from nuclear factor κ B abnormal expression. Preliminary data also demonstrate cerebro- and hepatoprotecting properties of mildronate in AZT-toxicity models. We suggest that mildronate may target its action predominantly to mitochondria. The present study in isolated rat liver mitochondria was designed to clarify mitochondrial targets for mildronate by using AZT as a model compound. The aim of this study was to investigate: (1) whether mildronate may protect mitochondria from AZT-induced toxicity; and (2) which is the most critical target in mitochondrial processes that is responsible for mildronate's regulatory action. The results showed that mildronate protected mitochondria from AZT-induced damage predominantly at the level of complex I, mainly by reducing hydrogen peroxide generation. Significant protection of AZT-caused inhibition of uncoupled respiration, ADP to oxygen ratio, and transmembrane potential were also observed. Mildronate *per se* had no effect on the bioenergetics, oxidative stress, or permeability transition of rat liver mitochondria. Since mitochondrial complex I is the first enzyme of the respiratory electron transport chain and its damage is considered to be responsible for different mitochondrial diseases, we may account for mildronate's effectiveness in the prevention of pathologies associated with mitochondrial dysfunctions. Copyright © 2008 John Wiley & Sons, Ltd.

KEY WORDS — mildronate; AZT; mitochondria; respiratory complex I; rat liver

INTRODUCTION

Mitochondria play an essential role in the cell by providing energy and controlling cell survival.¹ Therefore, mitochondrial dysfunction can cause a wide range of diseases, such as diabetes and obesity,² neurodegenerative diseases, such as Parkinson's and Alzheimer's,³ as well as ophtalmoplegia⁴ and

myopathy.⁵ Furthermore, hundreds of mitochondrial proteins vary in amounts from tissue to tissue, suggesting that the control of mitochondrial gene activity is very complex.⁶ The main causes of mitochondrial dysfunction are mutations of mitochondrial DNA³ and interruption of the homeostatic loop between mitochondria and the reactive oxygen species generated by mitochondrial respiratory chain activity.⁷

Supporting the strategy that mitochondrial dysfunction may be protected/reversed by drugs capable of regulating and improving mitochondrial processes,⁸ we suggested that mildronate, a drug of aza-butYRObetaine

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class, may target mitochondria. This suggestion is based on previous data showing that mildronate is capable of protecting mitochondrial membranes from damage by free fatty acids (FFAs),⁹ as well as from AZT-induced morphological changes and activation of nuclear factor κ Bp65 in mice cardiac tissue.¹⁰

Furthermore, it was also shown that mildronate decreases the concentration of lactic acid and increases ATP level in cardiac tissue after coronary artery occlusion;¹¹ as well as preventing the drop of ATP content during hypoxia of isolated rat heart.¹² A plausible mechanism to explain mildronate's protective action is related to its ability to regulate carnitine levels by acting as a competitive inhibitor of gamma-butyrobetaine (GBB) hydroxylase,^{13,14} thus inhibiting GBB transformation to carnitine. In fact, in rat liver mitochondria, mildronate was found to inhibit carnitine-palmitoyltransferase-1, thus protecting against long-chain fatty acid accumulation and subsequent damage of mitochondrial membranes.⁹

In order to gain some insight into understanding of mildronate's mechanism of action, particularly at the level of mitochondrial functions, we investigated its influence on azidothymidine (AZT) hepatotoxicity, using this drug as a mitochondria-compromising agent. AZT is a well-known anti-HIV drug of nucleoside reverse transcriptase class, used as one of the main drug components of highly active anti-retroviral therapy (HAART). The toxic influences on mitochondria are widely described: AZT depleted mitochondrial DNA in isolated rat liver and heart mitochondria,¹⁵ caused dysfunction of mitochondrial respiratory chain, leading to anaerobic ATP synthesis and lactic acid production,¹⁶ histopathological and ultrastructural changes of mitochondria,¹⁷ and oxidative stress by activation of reactive oxygen species.¹⁸ These events are considered as the main causes leading to severe side effects such as hepatotoxicity,¹⁹ cardiac and skeletal muscle pathologies, peripheral neuropathy, pancreatitis, lactic acidosis, and bone marrow suppression.¹⁸

In the present study, we examined the influence of mildronate in the AZT-toxicity model of isolated rat liver mitochondria.

MATERIALS AND METHODS

Chemicals

The chemicals were from Sigma Chemical Company (St Louis, MO, USA): ADP, rotenone (inhibitor of complex I), *p*-trifluoromethoxyphenylhydrazone

(FCCP, uncoupler), antimycin A (inhibitor of complex III), cyclosporine A (inhibitor of mitochondrial permeability transition (MPT)), catalase (anti-oxidant enzyme), components of media. Azidothymidine (3'-azido-3'-deoxythymidine, AZT, zidovudine, retrovir*, 100 mg capsules) from Wellcome Foundation Ltd., London; mildronate [3-(2,2,2-trimethylhydrazinium) propionate dihydrate] from the Joint Stock Company "Grindex" (Riga, Latvia).

A solution of AZT was prepared as follows: the content of one 100 mg capsule of AZT was suspended in 5 ml of H₂O in a glass centrifuge tube overnight. Then, the suspension was centrifuged at 3000 rpm for 10 min, the supernatant was saved and stored in aliquots of 2 ml each in the cold (4°C). The presence of AZT in the supernatant was confirmed by UV spectra (200–400 nm) and its concentration was calculated to be 50 mM using a molar extinction coefficient (226 nm) of 11 650.²⁰ Mildronate was dissolved in 5 ml of distilled water to obtain 50 mM solution. The concentrations of the drugs used in this study were 0.1, 0.2, 0.5, 1.5, 2.5, 5, and 10 μ mol mg⁻¹ protein.

Animals

Male Wistar rats (250–350 g), were obtained from Central Vivarium, Faculty of Medicine, University of Coimbra, Coimbra, Portugal, housed at 22 ± 2°C under artificial light with a 12 h light/dark cycle and with access to water and food *ad libitum*. The experiments were carried out in accordance with the National Requirements for Vertebrate Animal Research and with the European Convention for the Protection of Animals used for Experimental and other Scientific Purposes.

Isolation of rat liver mitochondria

Rat liver mitochondria were isolated from male 6-week-old Wistar rats by differential centrifugation according to conventional methods.²¹ After washing, the pellet was gently re-suspended in the washing medium at a protein concentration of about 50 mg ml⁻¹. Protein content was determined by the biuret method,²² using bovine serum albumin as a standard.

Measurement of respiratory activities

Oxygen consumption was monitored polarographically with a Clark-type electrode, in a closed glass

chamber equipped with magnetic stirring, thermostated at 30°C. Mitochondria (1 mg protein) were incubated in 1 ml of medium containing 250 mM sucrose, 10 mM Hepes (pH 7.2), 20 mM KCl, 5 mM K₂HPO₄, and 2 mM MgCl₂, supplemented with 2 μM rotenone (only when succinate was used as respiratory substrate), in the presence and absence of AZT, mildronate, and mildronate + AZT, then energized with either 10 mM glutamate/5 mM malate or 10 mM succinate. To induce state 3 respiration, ADP (100–200 nmol mg⁻¹ protein) was added. FCCP-stimulated respiration (uncoupled respiration) was initiated by the addition of 1 μM FCCP. Respiration rates were calculated by assuming that the saturation oxygen concentration was 230 nmol O₂ ml⁻¹. Control values are expressed in nmol O₂ per mg protein per minute. The respiratory control ratio (RCR), which is calculated by the ratio between state 3 (consumption of oxygen in the presence of substrate and ADP) and state 4 (consumption of oxygen after ADP phosphorylation), is an indicator of mitochondrial membrane integrity. ADP/oxygen ratio (ADP/O) is expressed as the ratio between the amount of the added ADP and the consumed oxygen during state-3 respiration, and served as index of oxidative phosphorylation efficiency. Both RCR and ADP/O were calculated according to a previously described method.²³

Measurement of mitochondrial transmembrane potential

The mitochondrial transmembrane potential ($\Delta\psi$) was measured indirectly based on the activity of the lipophilic cation tetraphenylphosphonium (TPP⁺) using a TPP⁺-selective electrode, as previously described.²⁴ Mitochondria (1 mg protein) were incubated at 30°C, in 1 ml of medium containing 250 mM sucrose, 10 mM Hepes (pH 7.2), 20 mM KCl, 5 mM K₂HPO₄, and 2 mM MgCl₂, supplemented with 3 μM TPP⁺ and 2 μM rotenone (when using succinate as respiratory substrate), in the absence and presence of AZT, mildronate, and mildronate + AZT, then energized with either 10 mM glutamate/5 mM malate or 10 mM succinate. To induce phosphorylation, ADP (100–200 nmol mg⁻¹ protein) was added. No correction was made for the “passive” binding of TPP⁺ to the mitochondria membranes because the purpose of the experiments was to show relative changes in potential rather than absolute values. Phosphorylation time is considered as the time required by mitochondria to phosphorylate all the ADP added. It was

calculated by measuring the time that is needed for restoration of the membrane potential after a pulse of ADP by taking into account the recording speed.

Ca²⁺-induced mitochondrial permeability transition (MPT)

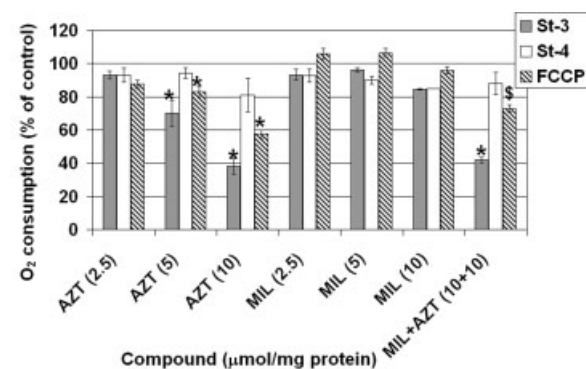
To detect Ca²⁺-induced MPT, we used oxygen and TPP⁺ electrodes and a medium containing 200 mM sucrose, 10 mM Hepes-Tris (pH 7.4), 1 mM KH₂PO₄, 10 μM EGTA, supplemented with 2 μM rotenone, in the absence and presence of the test compounds. Mitochondria (1 mg), incubated at 30°C in 1 ml of medium, were energized with 5 mM succinate and three additions of 20 nmol Ca²⁺ mg⁻¹ protein each were used to induce MPT.²⁵ Control assays, in both the absence and presence of Ca²⁺ + 1 μM cyclosporin A (CsA), were also performed.

Hydrogen peroxide generation

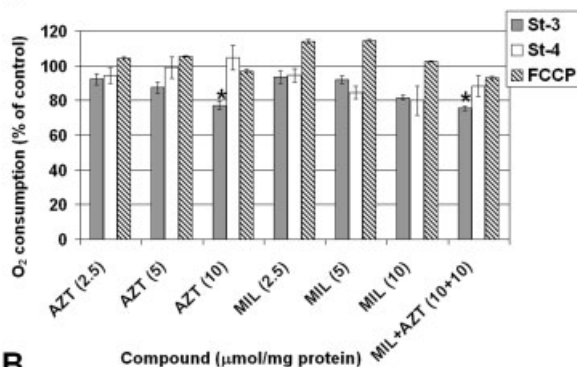
Hydrogen peroxide (H₂O₂) generation was measured fluorometrically using a modification of the method previously described.²⁶ Briefly, mitochondria (0.375 mg) were incubated at 30°C in 1.5 ml of phosphate buffer, pH 7.4, containing 5 mM phosphate, 0.1 mM EGTA, 145 mM KCl, 30 mM Hepes, 0.1 mM homovallinic acid, and 6 U ml⁻¹ horseradish peroxidase (in the presence or absence of inhibitors such as rotenone, antimycin A, catalase, and the test drugs). The reactions were started by adding 10 mM glutamate/5 mM malate or 10 mM succinate and stopped at 15 min with 0.5 ml of cold 0.1 M glycine-NaOH (pH 12) containing 25 mM EDTA. The mitochondrial suspensions were centrifuged at 850 g for 10 min. The enzyme catalase was used as a positive control to confirm that the generated H₂O₂ is scavenged. The fluorescence of supernatants was measured at 312 nm as excitation and 420 nm as emission wavelengths. The peroxide generation was calculated using a standard curve of H₂O₂. The standards and samples were incubated under the same conditions.

Statistical analysis

All the experiments were performed using four independent mitochondrial preparations. The values were expressed as mean ± SEM. Means were compared using one-way ANOVA, post-test Dunnett. Statistical significance was set at $p < 0.05$.



A



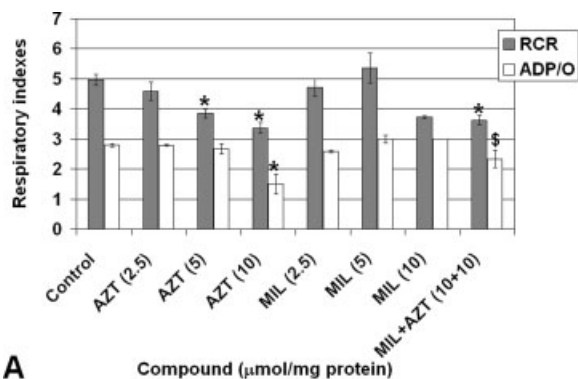
B

Figure 1. Effects of azidothymidine (AZT), mildronate (MIL), and MIL + AZT on glutamate/malate- (A) and succinate-supported (B) O_2 consumption in state 3, state 4, and FCCP-uncoupled respiration of rat liver mitochondria. Mitochondria were incubated under standard conditions as described in Section "Materials and Methods" without (control) or with the indicated concentrations of AZT, MIL, and MIL + AZT. Control values expressed in nmol O_2 per mg protein per minute: (A) state 3 = 46.72 ± 1.54 ; state 4 = 9.36 ± 1.01 ; FCCP = 22.07 ± 2.47 ; (B) state 3 = 71.34 ± 0.56 ; state 4 = 14.35 ± 0.84 ; FCCP = 68.43 ± 2.41 . Results correspond to the mean \pm SEM of four experiments obtained from different mitochondrial preparations. * $p < 0.05$ versus control, $^{\$}p < 0.05$ versus AZT $10 \mu\text{mol mg}^{-1}$ protein

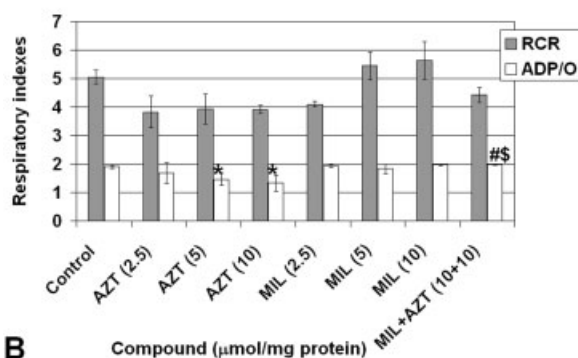
RESULTS

Effects of AZT, mildronate, and mildronate + AZT on mitochondrial bioenergetics

The effects of AZT, mildronate, and AZT + mildronate on glutamate/malate- and succinate-supported respiratory rates characteristic of state 4, state 3 and uncoupled respiration, as well as, respiratory indices RCR and ADP/O ratio of rat liver mitochondria *in vitro* are depicted in Figures 1 and 2. State 4 respiration was not affected using either of the respiratory substrates, whereas state 3 respiration was



A



B

Figure 2. Effects of azidothymidine (AZT), mildronate (MIL), and MIL + AZT on glutamate/malate- (A) and succinate-supported (B) respiration indices: respiratory control ratio (RCR) and ADP/O ratio of rat liver mitochondria without (control) or with the indicated concentrations of AZT, MIL, and MIL + AZT. Mitochondria were incubated under standard conditions as described in Section "Materials and Methods". Results correspond to the mean \pm SEM of four experiments obtained from different mitochondrial preparations. * $p < 0.05$ versus control, $^{\#}p < 0.05$ versus AZT $5 \mu\text{mol mg}^{-1}$ protein, $^{\$}p < 0.05$ versus AZT $10 \mu\text{mol mg}^{-1}$ protein

inhibited by AZT ($10 \mu\text{mol mg}^{-1}$ protein) by 62 and 23% with glutamate/malate and succinate, respectively. Uncoupled respiration supported by glutamate/malate was also significantly inhibited (by 45%) with $10 \mu\text{mol mg}^{-1}$ protein AZT, whereas uncoupled respiration supported by succinate was less affected (by 3%). At the same concentration, AZT significantly depressed RCR (from about 5 to 3) with glutamate/malate but not with succinate. The ADP/O ratio was affected significantly more with glutamate/malate (46%) than with succinate (23%) as substrate. In contrast to AZT, mildronate concentrations up to $10 \mu\text{mol mg}^{-1}$ protein, did not affect any of the parameters reported above using either glutamate/malate or succinate as the respiratory substrates.

Combined administration of mildronate with AZT (both $10 \mu\text{mol mg}^{-1}$ protein) prevented AZT-induced inhibition of uncoupled respiration and ADP/O with glutamate/malate, as well as ADP/O depression with succinate.

The effects of AZT, mildronate, and mildronate + AZT on mitochondrial transmembrane potential ($\Delta\Psi$) and phosphorylation time supported by glutamate/malate and succinate are shown in Figure 3 and Table 1. AZT dissipated $\Delta\Psi$ and increased the

phosphorylation time for both substrates, but these effects were more pronounced with glutamate/malate (from about -208 to -196 mV) than with succinate (from about -215 to -208 mV). The phosphorylation time was increased by 77% for glutamate/malate (Figure 3A and Table 1) and by 36% for succinate (Figure 3B and Table 1). Mildronate did not significantly affect $\Delta\Psi$ and phosphorylation time dependent on glutamate/malate or succinate. Combined administration of mildronate with AZT (both

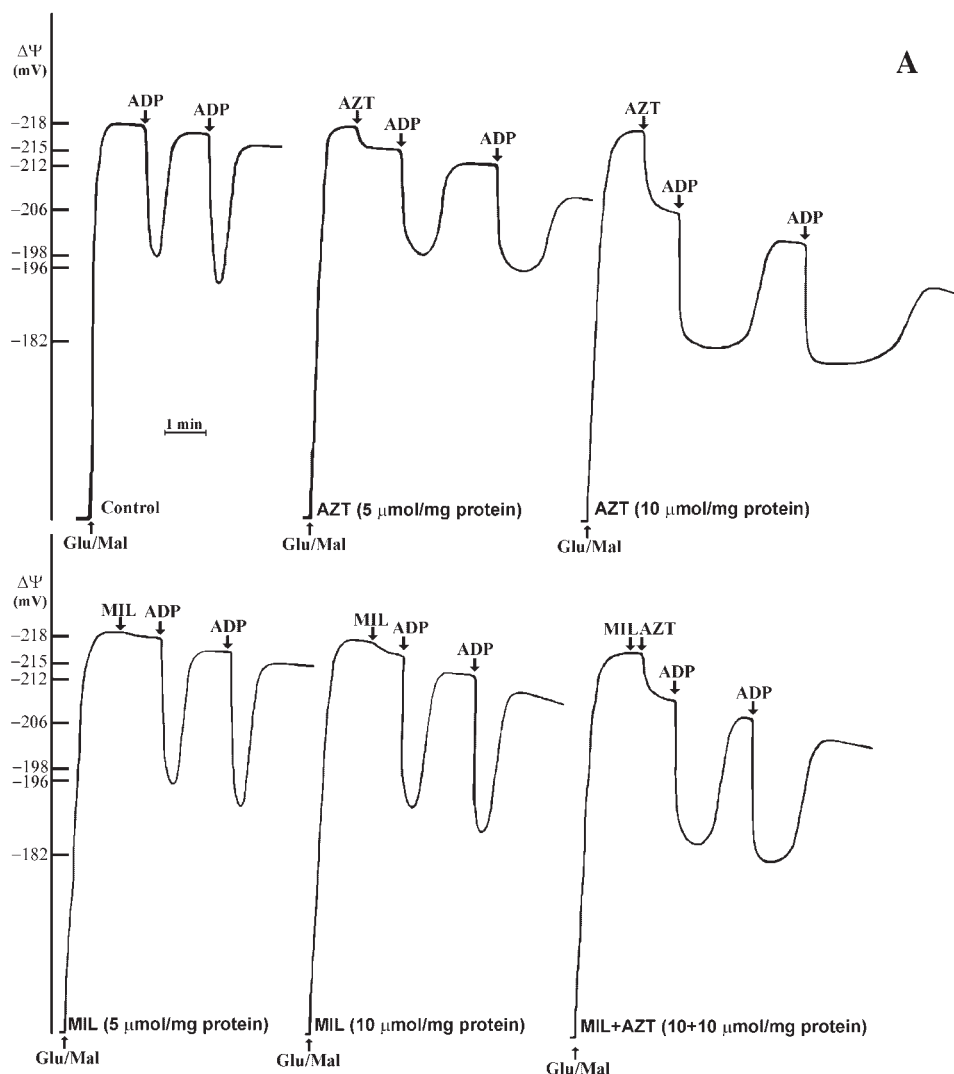


Figure 3. Effects of azidothymidine (AZT), mildronate (MIL), and MIL + AZT on glutamate/malate- (A) and succinate-supported (B) transmembrane potential ($\Delta\Psi$). Rat liver mitochondria were incubated in reaction medium as described in Section "Materials and Methods" without (control) or with the indicated concentrations of AZT, MIL, and MIL + AZT. Reactions were started with 10 mM glutamate/5 mM malate or 10 mM succinate. 100 nmol mg^{-1} protein ADP was added to induce phosphorylation. The traces represent typical direct recordings representative of four experiments obtained from different mitochondrial preparations

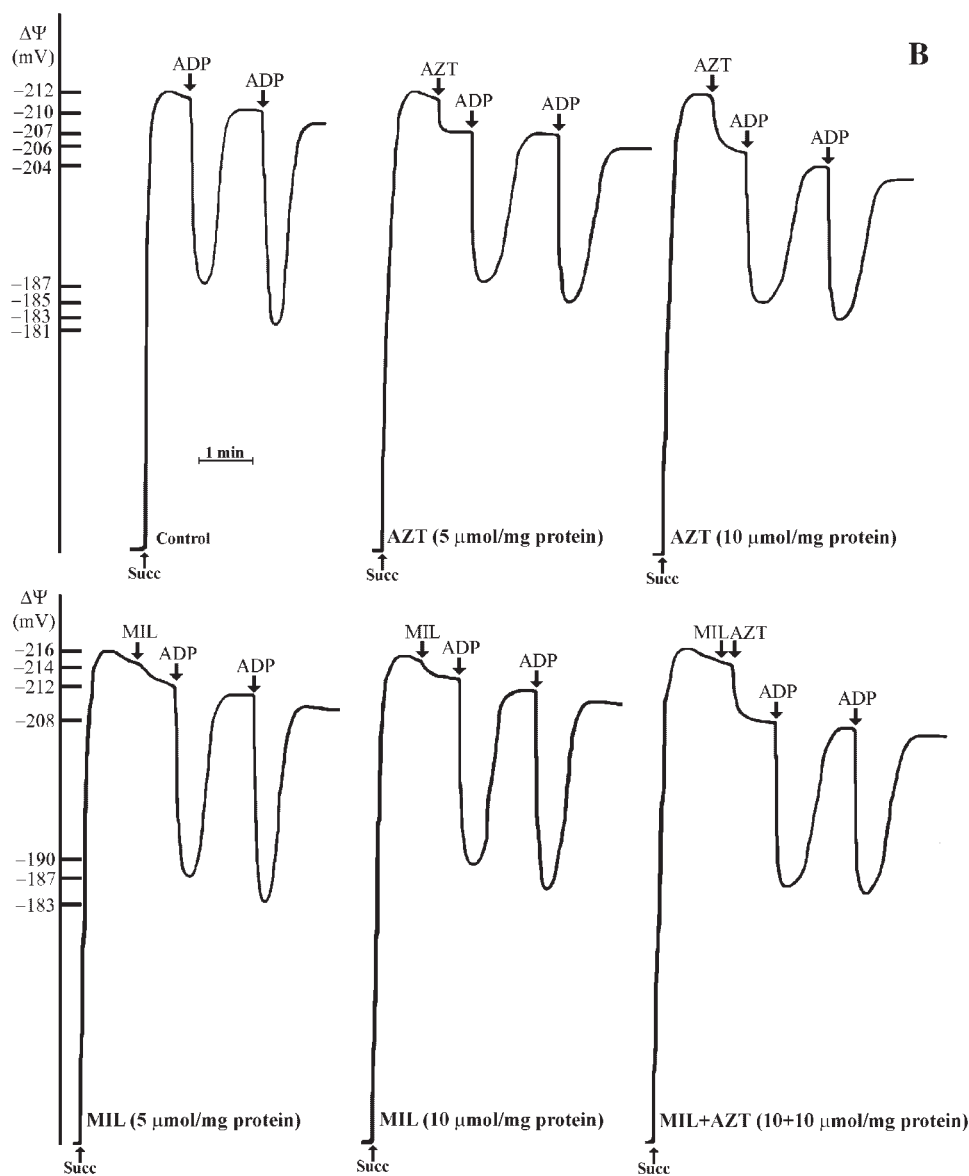


Figure 3. (Continued)

$10 \mu\text{mol mg}^{-1}$ protein) prevented AZT-induced inhibition of repolarization with glutamate/malate (Figure 3A and Table 1).

Effects of AZT, mildronate, and mildronate + AZT on hydrogen peroxide generation

The effects of AZT, mildronate, and mildronate + AZT on hydrogen peroxide generation by rat liver mitochondria *in vitro* were studied using both

glutamate/malate and succinate as respiratory substrates (Figure 4). In the absence of mildronate and AZT, the H_2O_2 generated by glutamate/malate-respiring mitochondria significantly increased when mitochondria were treated with rotenone (inhibitor of complex I) alone and with rotenone + antimycin A (inhibitor of complex III, Figure 4A). The elevated production of H_2O_2 was reduced by catalase to levels below those observed under basal conditions (mitochondria in the presence of glutamate/malate), as

Table 1. Effects of AZT (azidothymidine), MIL (mildronate), and AZT + MIL on glutamate/malate- (A) or succinate-dependent (B) transmembrane potential ($\Delta\Psi$) and phosphorylation of rat liver mitochondria at the different indicated situations

Compound ($\mu\text{mol mg}^{-1}$ protein)	$\Delta\Psi$ (mV)			Phosphorylation time (s)
	Glutamate/malate energization	ADP depolarization	Repolarization	
Control (0)	-212.9 \pm 1.2	-24.2 \pm 0.9	-206.0 \pm 1.4	40.5 \pm 1.0
AZT (2.5)	-218.5 \pm 0.5	-23.2 \pm 1.2	-209.8 \pm 2.0	44.0 \pm 1.5
AZT (5)	-209.0 \pm 7.4	-21.5 \pm 0.5	-191.0 \pm 3.0*	73.5 \pm 11.9*
AZT (10)	-202.0 \pm 6.0	-24.8 \pm 3.6	-182.0 \pm 5.2*	172.5 \pm 13.4*
MIL (2.5)	-216.5 \pm 0.5	-22.3 \pm 3.6	-211.8 \pm 0.5	32.1 \pm 2.0
MIL (5)	-214.4 \pm 2.5	-25.5 \pm 1.5	-208.0 \pm 1.0	39.0 \pm 1.5
MIL (10)	-212.5 \pm 0.5	-26.4 \pm 0.9	-207.5 \pm 1.5	39.8 \pm 6.8
AZT + MIL (10 + 10)	-212.0 \pm 1.0	-26.7 \pm 0.4	-200.3 \pm 1.3* [§]	98.6 \pm 6.7

Compound ($\mu\text{mol mg}^{-1}$ protein)	$\Delta\Psi$ (mV)			Phosphorylation time (s)
	Succinate energization	ADP depolarization	Repolarization	
Control (0)	-213.9 \pm 0.6	-21.3 \pm 0.9	-212.5 \pm 0.7	41.3 \pm 3.2
AZT (2.5)	-213.0 \pm 1.4	-19.6 \pm 0.7	-210.6 \pm 0.7*	41.3 \pm 3.8
AZT (5)	-219.0 \pm 2.0	-19.5 \pm 0.6	-208.8 \pm 0.9*	53.3 \pm 0.8
AZT (10)	-214.5 \pm 0.5	-17.7 \pm 1.3	-205.3 \pm 0.6*	64.5 \pm 3.8*
MIL (2.5)	-214.3 \pm 1.8	-18.9 \pm 1.2	-212.7 \pm 0.9	34.5 \pm 1.5
MIL (5)	-221.0 \pm 1.0*	-22.7 \pm 1.2	-216.8 \pm 0.5*	42.0 \pm 3.0
MIL (10)	-217.0 \pm 3.0	-22.5 \pm 1.2	-212.3 \pm 1.9	51.0 \pm 3.2
AZT + MIL (10 + 10)	-214.0 \pm 1.0	-21.0 \pm 1.4	-204.3 \pm 0.5*	57.0 \pm 1.2*

Rat liver mitochondria were incubated in reaction medium without (control) or with the indicated concentrations of AZT, MIL, and AZT + MIL. The results are mean values \pm SEM of four independent experiments. * p < 0.05 versus control; # p < 0.05 versus AZT 5 $\mu\text{mol mg}^{-1}$ protein; § p < 0.05 versus AZT 10 $\mu\text{mol mg}^{-1}$ protein.

expected. AZT concentrations up to 10 $\mu\text{mol mg}^{-1}$ protein significantly increased H_2O_2 generation by glutamate/malate-respiring mitochondria in the absence and presence of rotenone, but were without significant effect in the presence of rotenone + antimycin A. A maximal increase of H_2O_2 generation (of about 70%) was observed at 10 $\mu\text{mol mg}^{-1}$ protein AZT for both conditions. Mildronate concentrations up to 10 $\mu\text{mol mg}^{-1}$ protein did not significantly affect H_2O_2 generation under any of the conditions used (Figure 4A). However, co-administration of mildronate with AZT (both 10 $\mu\text{mol mg}^{-1}$ protein) significantly reduced H_2O_2 generation promoted by AZT alone either under basal conditions (by about 55%) or in mitochondria treated with rotenone (by about 34%, Figure 4A). Similar to glutamate/malate, succinate-respiring mitochondria (in the presence of rotenone, Figure 4B) responded to treatment with antimycin A with maximally stimulated H_2O_2 production. This effect was reduced by catalase to levels below those observed under basal conditions (mitochondria in the presence of rotenone). A maximal increase of H_2O_2 generation (by about 58%) was

observed at 10 $\mu\text{mol mg}^{-1}$ protein AZT with succinate-respiring mitochondria under basal conditions. No effect was noted in mitochondria treated with rotenone + antimycin A (Figure 4B). Mildronate concentrations up to 10 $\mu\text{mol mg}^{-1}$ protein did not significantly affect the H_2O_2 generation either under basal conditions or in mitochondria treated with rotenone + antimycin A (Figure 4B). In contrast to glutamate/malate-respiring mitochondria, combined administration of mildronate and AZT (both 10 $\mu\text{mol mg}^{-1}$ protein) did not significantly reduce H_2O_2 generation promoted by AZT alone in succinate-respiring mitochondria (basal conditions).

Effects of AZT, mildronate, and mildronate + AZT on Ca^{2+} -induced mitochondrial permeability transition (MPT)

Figure 5 shows the effects of AZT, mildronate, and mildronate + AZT on the MPT *in vitro*. This effect was evaluated by measuring the decline in mitochondrial

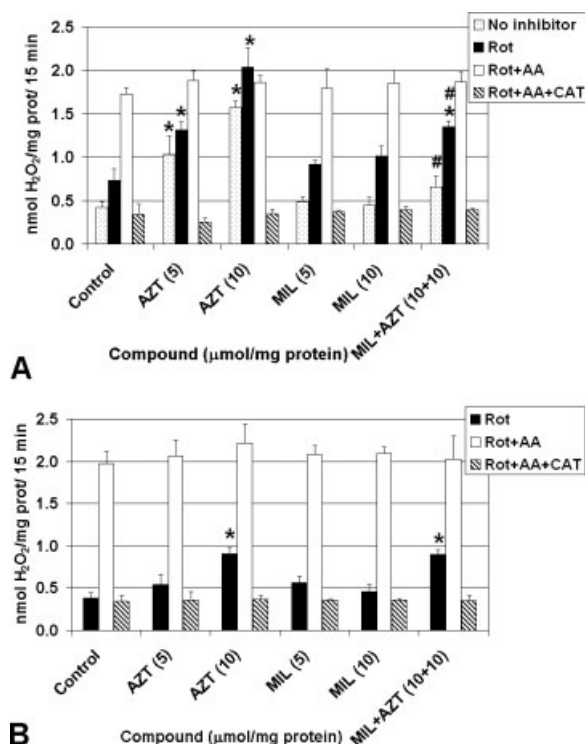


Figure 4. Effect of azidothymidine (AZT), mildronate (MIL), and MIL + AZT on hydrogen peroxide production of rat liver mitochondria. Mitochondria were incubated under standard conditions as described in Section "Materials and Methods" with glutamate/malate (A) or succinate (B) as respiratory substrates under the following conditions: without inhibitors (dotted bars); with 2 μM rotenone (Rot, black bars); rotenone and 10 μM antimycin A (Rot + AA, white bars); and rotenone, antimycin A and 643 U ml⁻¹ catalase (Rot + AA + CAT, dashed bars). The results correspond to the mean ± SEM of four experiments obtained from different mitochondrial preparations. **p* < 0.05 versus control, #*p* < 0.05 versus AZT 10 μmol mg⁻¹ protein

transmembrane potential ($\Delta\Psi$) and the increase in oxygen consumption which are typical phenomena that follow the induction of MPT. Under control conditions, the addition of 5 mM succinate produced a $\Delta\Psi$ of ≈ -217 mV (negative inside mitochondria), corresponding to the respiratory state 4. The first pulse of 20 nmol Ca²⁺ mg⁻¹ protein led to a rapid depolarization (decrease of $\Delta\Psi$) followed by a small repolarization (recovery of $\Delta\Psi$). The depolarization was due to the entry of Ca²⁺ into the electronegative mitochondrial matrix, followed by the efflux of H⁺ for restoring the $\Delta\Psi$. Consequently, respiratory state 4 increased from 13 to 17 nmol O₂ mg⁻¹ protein min⁻¹. However, a second and third pulse of 20 nmol Ca²⁺ mg⁻¹ protein led to a total depolarization of mitochondria and to a maximal

increase of respiratory state 4 (60 nmol O₂ min⁻¹ mg⁻¹ protein).

Incubation of mitochondria with AZT concentrations up to 5 μmol mg⁻¹ protein or with mildronate concentrations up to 10 μmol mg⁻¹ protein, for 2 min before energizing with succinate, did not affect significantly the capacity of mitochondria to accumulate Ca²⁺ (Figure 5). However, at concentrations 5–10 μmol mg⁻¹ protein AZT reduced the capacity of mitochondria to accumulate Ca²⁺ to 40 nmol mg⁻¹ protein. Mildronate, in combination with AZT, reversed this effect to control values (60 nmol mg⁻¹ protein, Figure 5). Incubation of mitochondria with 0.85 μM cyclosporin A, a specific inhibitor of MPT,²⁷ for 2 min before energizing with succinate, either in the absence or presence of AZT or mildronate totally blocked mitochondrial depolarization and increased respiratory state 4 (results not shown), showing that these effects have been induced by MPT.

DISCUSSION

This study was devoted to the clarification of the mechanism of action of mildronate (MET-88), an aza-butyrobetaine derivative, which was previously shown as a cytoprotective agent capable of regulating the cardiovascular system and cardiac functions.^{11,12} It is suggested that mildronate's protective action is based on its ability to inhibit carnitine palmitoyltransferase-1 activity thus decreasing the amount of activated FFAs in mitochondria.⁹ Since activated FFAs possess detergent-like activity damaging mitochondrial membranes, we suggest that mitochondria may serve as the target for mildronate's cytoprotective action. Thus, for the present study we have chosen the model compound AZT, a well-known mitochondria-compromising anti-HIV drug. Previously, several studies have demonstrated that AZT causes changes in mitochondrial structure, alters respiratory chain enzyme activity, lowers mitochondrial ATP, activates reactive oxygen species, increases lactic acid production, and causes loss of mitochondrial DNA and cytochrome C.^{28–30} Our previous experiments in AZT-induced cardiotoxicity model in mice,¹⁰ as well as preliminary studies in neuro- and hepatotoxicity states (unpublished data) demonstrated high anti-inflammatory and anti-apoptotic effects of mildronate, suggesting that it may regulate cellular damages initiated by AZT at least in part at the level of mitochondria.

Our present study was performed in an isolated rat liver mitochondria. The results obtained have provided evidence that mildronate (10 μmol mg⁻¹ protein)

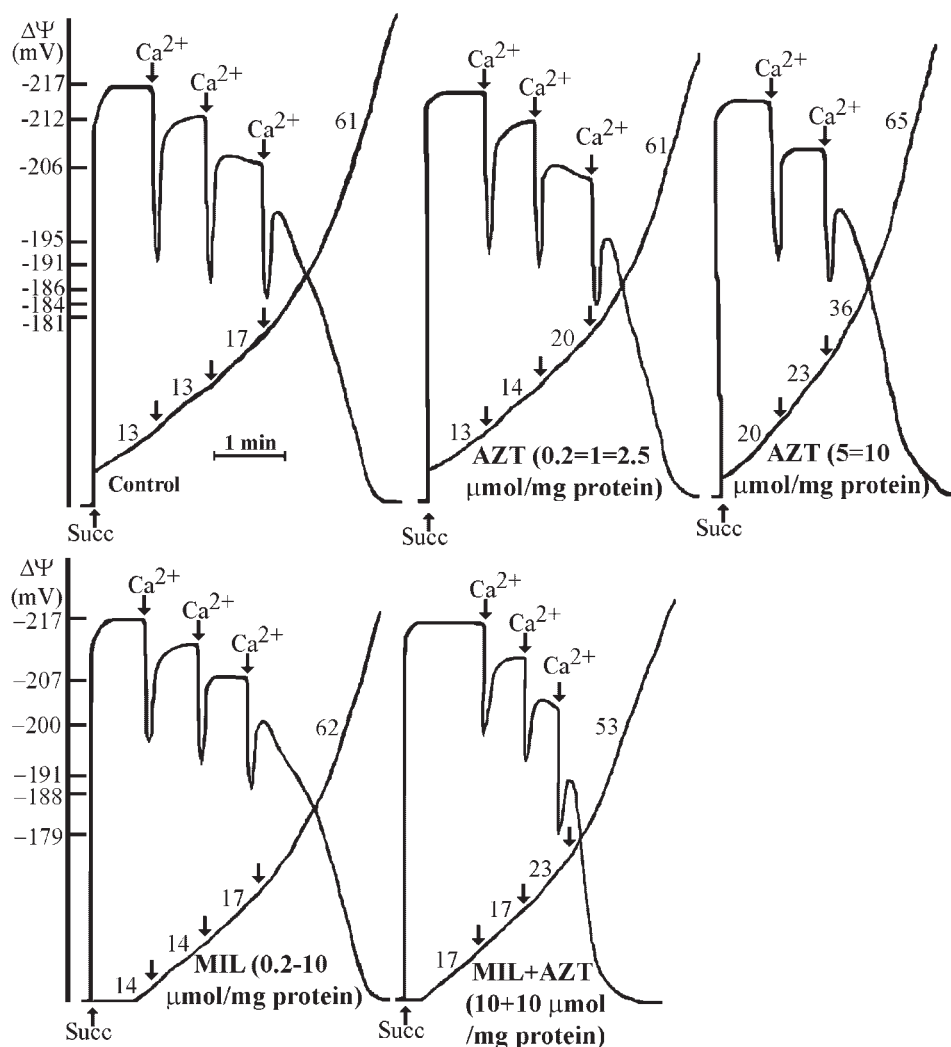


Figure 5. Effects of azidothymidine (AZT), mildronate (MIL), and MIL + AZT at indicated concentrations on mitochondrial permeability transition pore induction: susceptibility to Ca^{2+} (20 nmol mg^{-1} protein) addition. Freshly isolated rat liver mitochondria (1 mg) in 1 ml of standard medium supplemented with $3 \mu\text{M TPP}^+$ were energized with 5 mM succinate (as described in Section "Materials and Methods"). The traces represent typical direct recordings representative of four experiments obtained from different mitochondrial preparations

prevented AZT-induced mitochondrial toxicity. It remarkably reduced AZT-caused increase in production of H_2O_2 by 58% in basal conditions and by 34% in the presence of rotenone, an inhibitor of complex I. Since AZT-induced H_2O_2 production also under basal conditions, thus showing pro-oxidant properties, we suggest that the ability of attenuating the AZT effect may be provided via mildronate's anti-oxidant properties. Our data are in good agreement with other results

reporting that mildronate may protect heart tissue against the H_2O_2 -induced derangement in energy metabolism.³¹ Our data also showed that mildronate prevented the AZT-induced inhibition of uncoupled respiration and ADP/O ratio, and recovery of transmembrane potential (by 21, 36, and 9%, respectively) with glutamate/malate, thus providing the electron flow in the respiratory chain at the level of complex I, with subsequent improvement of the efficiency of state 3

respiration, RCR, mitochondrial transmembrane potential, and phosphorylation. However, in our experiments AZT-induced MPT was weakly expressed (induction by 33%); mildronate normalized this effect to control levels. Nevertheless, since it is known that activation of MPT compromises the normal integrity of the mitochondrial outer membrane, thus influencing inner membrane leading to the release of cytochrome C, activation of caspase family proteins and subsequent apoptosis, as well as metabolic changes resulting in subsequent necrosis,³² one may suggest that mildronate influences oxidative stress. Moreover, our previous data¹⁰ obtained in mice cardiac tissue demonstrated that mildronate protected AZT-increased expression of redox-sensitive nuclear transcription factor κ Bp65 (NF- κ Bp65), which can be activated by reactive oxygen species, and plays a critical role in immune and inflammatory responses.³³ Other data showed that mildronate improved cardiac sarcoplasmic reticulum Ca^{2+} uptake activity in rats with congestive heart failure following myocardial infarction.¹¹ Therefore, previous and present data demonstrating mildronate's activity to inhibit H_2O_2 production and MPT pore opening indicate mildronate's ability to halt apoptotic and necrotic processes at their early stages. It must be also noted that mildronate *per se* did not affect mitochondrial function either in glutamate/malate or succinate respiration.

In our experiments, we were faced with the fact that AZT caused strong alterations in mitochondrial function at relatively high concentrations of the range of 5–10 $\mu\text{mol mg}^{-1}$ protein. It inhibited bioenergetics (namely uncoupled respiration, respiration in state 3, RCR, ADP/O ratio, mitochondrial transmembrane potential, and phosphorylation) and considerably (by 44–64% compared to control) increased H_2O_2 production with glutamate/malate (respiratory substrate for complex I), indicating AZT's severe effect at the level of complex I. At the highest concentration used (10 $\mu\text{mol mg}^{-1}$ protein), AZT also increased H_2O_2 production when succinate was used as substrate, thus showing the influence of AZT on complex II. AZT had no effect on complex III, since it did not affect H_2O_2 formation in mitochondria inhibited with rotenone (inhibitor of complex I) + antimycin A (inhibitor of complex III). Our data are in good agreement with findings obtained in other studies which revealed inhibitory effects of AZT at concentrations 5–15 mM on bioenergetics at the level of complex I in rat liver, kidney, skeletal, and heart muscle mitochondria.³⁴ AZT had no effect at the level of complex II on mitochondrial bioenergetics of rat liver and kidney,³⁴ and heart.³⁵ AZT also induced an increase in H_2O_2

production in rat peritoneal macrophages,³⁶ and in AZT-treated mice muscle mitochondria,³⁷ as well as inhibition of NADH cytochrome C reductase, an enzyme of complex I.^{38,39} Nevertheless, in rat heart mitochondria, different data were obtained: no influence on bioenergetics and Ca^{2+} loading capacity at 0.01 mM, while inhibition of glutamate/malate-dependent RCR with corresponding increase in the rate of state 4 at 0.1 mM were found.³⁵ Recently, it was shown that the AZT inhibitory effect on complex I is due to the inhibition of cAMP-dependent phosphorylation of complex I.⁴⁰

We suggest that the high AZT concentrations found to be active in our mitochondrial studies can be considered as relevant, since AZT is used for the treatment of HIV-infected AIDS patients at doses of about 500–1500 mg daily for a long survival period.⁴¹ The same concerns apply to mildronate, which in the present study was protective at concentrations 5–10 $\mu\text{mol mg}^{-1}$ protein, and its clinical doses are known to amount to 1000 mg daily used at least for a 3-month period.⁴²

In conclusion, our present data have demonstrated for the first time that mildronate, a representative of the aza-butyrobetaine class, may protect the AZT-induced H_2O_2 formation in isolated rat liver mitochondria at the level of complex I, indicating mildronate as a mitochondria-targeted drug. Also, its anti-oxidant properties are essential for halting reactive oxygen species-induced oxidative stress reactions leading to cell death. Since mitochondrial complex I is the first enzyme of the respiratory electron transport chain and its deterioration is considered as a crucial cause for neurodegenerative diseases, such as Parkinson's and Alzheimer's, as well as diabetes type II, anti-HIV therapy-induced mitochondrial disorders, and others, one may suggest mildronate's usefulness in the treatment of different mitochondrial diseases.

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