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Effects of 1,4-dihydropyridine derivatives (cerebrocrast, gammapyrone, glutapyrone, and diethone) on mitochondrial bioenergetics and oxidative stress: a comparative study

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Abstract

The potential protective action of 1,4-dihydropyridine derivatives (cerebrocrast, gammapyrone, glutapyrone, and diethone) against oxidative stress was assessed on mitochondrial bioenergetics, inner membrane anion channel (IMAC), Ca^{2+}-induced opening of the permeability transition pore (PTP), and oxidative damage induced by the oxidant pair adenosine diphosphate (ADP)/Fe^{2+} (lipid peroxidation) of mitochondria isolated from rat liver. By using succinate as the respiratory substrate, respiratory control ratio (RCR), ADP to oxygen ratio (ADP/O), state 3, state 4, and uncoupled respiration rates were not significantly affected by gammapyrone, glutapyrone, and diethone concentrations up to 100 μM. Cerebrocrast at concentrations higher than 25 μM depressed RCR, ADP/O, state 3, and uncoupled respiration rates, but increased three times state 4 respiration rate. The transmembrane potential ($\Delta\Psi$) and the phosphate carrier rate were also decreased. At concentrations lower than 25 μM, cerebrocrast inhibited the mitochondrial IMAC and partially prevented Ca^{2+}-induced opening of the mitochondrial PTP, whereas gammapyrone, glutapyrone, and diethone were without effect. Cerebrocrast, gammapyrone, and glutapyrone concentrations up to 100 μM did not affect ADP/Fe^{2+}-induced lipid peroxidation of rat liver mitochondria, while very low diethone concentrations (up to 5 μM) inhibited it in a dose-dependent manner, as measured by oxygen consumption and thiobarbituric acid reactive substances formation. Diethone also prevented $\Delta\Psi$ dissipation due to lipid peroxidation initiated by ADP/Fe^{2+}. It can be concluded that: none of the compounds interfere with mitochondrial bioenergetics at concentrations lower than 25 μM; cerebrocrast was the only compound that affected mitochondrial bioenergetics, but only for concentrations higher than 25 μM; at concentrations that did not affect mitochondrial bioenergetics (≤25 μM), only cerebrocrast inhibited the IMAC and partially prevented Ca^{2+}-induced opening of the PTP; diethone was the only compound that expressed antioxidant activity at very low concentrations (≤5 μM). Cerebrocrast acting as an inhibitor of the IMAC and diethone acting as an antioxidant could provide effective protective roles in preventing mitochondria from oxidative damage, favoring their therapeutic interest in the treatment of several pathological situations known to be associated with cellular oxidative stress.

Keywords: 1,4-Dihydropyridine derivatives; Cerebrocrast; Gammapyrone; Glutapyrone; Diethone; Liver mitochondria; Mitochondrial bioenergetics; Oxidative stress; Antioxidants; Mitochondrial permeability transition pore; Mitochondrial inner membrane anion channel

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1. Introduction

Cerebrocrast, gammapyrone, glutapyrone, and diethone are four new structurally and functionally different 1,4-dihydropyridine (DHP) derivatives developed at the Latvian Institute of Organic Synthesis, Riga, Latvia, and the molecular structure of these compounds is shown in Fig. 1. Cerebrocrast [IOS-1.1212; 2-propoxyethyl ester of 2,6-dimethyl-4-(2-difluoromethoxyphenyl)-1,4-dihydropyridine-3,5-dicarboxylic acid] is a neuroprotector and cognition enhancer compound (Misãne et al., 1993; Klusa, 1995; Velena et al., 1997; Klegeris et al., 2002). Its molecule is characterized by increased lipophilicity due to both medium length 2-propoxy-ethyl side chains with oxa atoms in positions 3 and 5 of the DHP ring and the combination with the difluoromethoxy-group at position 2 of the phenyl ring which is joined to the DHP at position 4 (Misãne et al., 1993; Klusa, 1995; Velena et al., 1997; Klegeris et al., 2002). It is considered that this lipophilic molecule is capable of penetrating easily the blood–brain barrier, the plasma and organelles membranes, including those of mitochondria (Misãne et al., 1993; Klusa, 1995; Velena et al., 1997; Klegeris et al., 2002). Gammapyrone [IOS-1.2134; 4-(2,6-dimethyl-3,5-diethoxycarbonyl-1,4-dihydropyridine-4-carboxamido) butyric acid] is a neuroprotector (Misãne et al., 1993), whereas glutapyrone [IOS-1.397; disodium 2-(2,6-dimethyl-3,5-diethoxycarbonyl-1,4-dihydropyridine-4-carboxamido) glutarate] is an antiarrhythmic compound (Karpova et al., 1993; Klusa et al., 1996; Velena et al., 1997; Briede et al., 2000). These two compounds are members from the family of a novel type of peptidomimetic DHP compounds (amino acid-containing DHPs), designed by adding neuroprotective amino acids (glutamic, gamma aminobutyric acid, and others) via the carbonyl group to the DHP ring in position 4. They differ from the classical DHP compounds by their high water solubility and low toxicity (Velena et al., 1997). Diethone ([1287-53-5]; 2,6-dimethyl-3,5-diethoxycarbonyl-1,4-dihydropyridine), a four-unsubstituted DHP molecule, is an antioxidant and radioprotector compound characterized

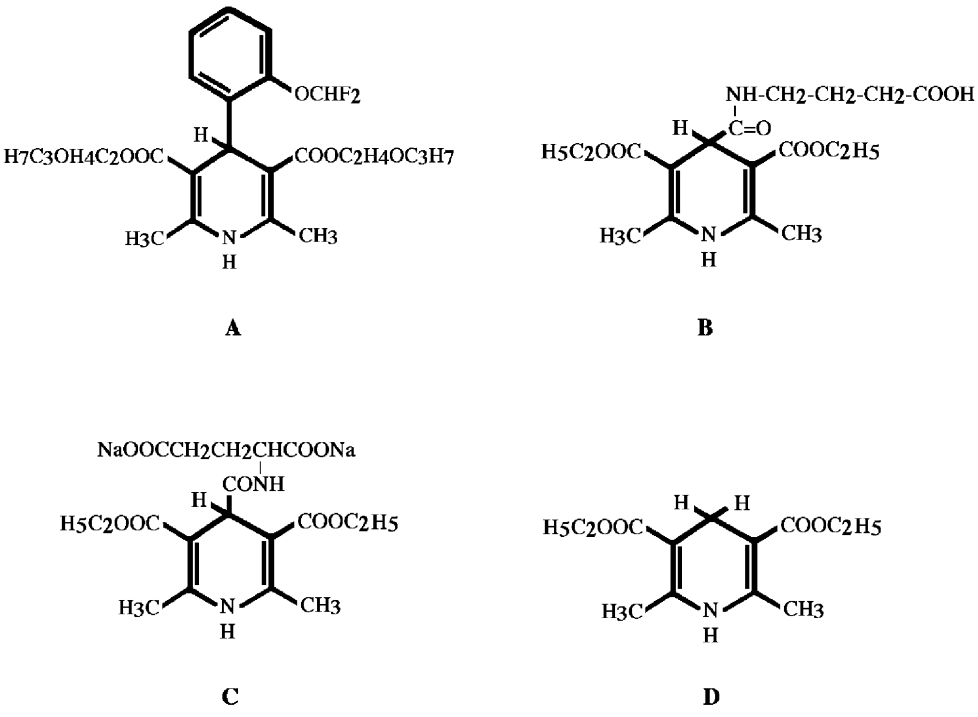

Fig. 1. Molecular structures of cerebrocrast (A), gammapyrone (B), glutapyrone (C), and diethone (D).

as being lipophilic and non-toxic (Ivanov et al., 1990; Abdalla et al., 1999; Velena et al., 1999).

Cerebrocrast, gammapyrone, and glutapyrone did not influence significantly the $^{45}Ca^{2+}$ uptake by rat cortical synaptosomes (Karpova et al., 1993; Misãne et al., 1993). Glutapyrone did not change the $^{45}Ca^{2+}$ uptake in rabbit myocardial slices or the contraction force of isolated spontaneously beating rabbit atria (Klusa et al., 1996). These data suggest that these compounds lack calcium antagonist properties that are typical of the traditional DHP (Misãne et al., 1993; Karpova et al., 1993; Klusa et al., 1996). No information exists in the literature concerning the diethone calcium antagonizing action. Due to their protective properties, these DHP derivatives may be potentially useful for the treatment of several pathological processes, including those associated with oxidative stress.

Oxidative stress refers to the cytotoxic effects of reactive oxygen species (ROS) which may damage cellular components including nucleic acids, proteins, and membrane phospholipids, promoting cell death (Kehrer, 1993; Halliwell and Gutteridge, 1994, 1999). Mitochondria, whose main function is ATP synthesis by oxidative phosphorylation, are also considered to be the major endogenous source of cellular ROS, under normal and pathological conditions, and perhaps of oxidative stress in general (Lee and Wei, 2000; Murphy and Smith, 2000; Pedersen, 2000). Mitochondria are also the cellular component most extensively affected by increased concentrations of ROS (Lee and Wei, 2000; Murphy and Smith, 2000; Pedersen, 2000). Therefore, the potential protective action of the DHP derivatives (cerebrocrast, gammapyrone, glutapyrone, and diethone) may contribute to the preservation of mitochondrial functions in several pathological conditions associated with oxidative stress. However, it is important to analyze the direct effect of these compounds on mitochondrial bioenergetics, since their interaction with mitochondrial membranes can disturb the coupling efficiency between oxidation and phosphorylation, promoting bioenergetic deficits leading to the loss of several functions vital to the survival of the cell and the organism (Wallace and Starkov, 2000).

The purpose of this study was to investigate, firstly, the direct effect of cerebrocrast, gammapyrone, glutapyrone, and diethone on mitochondrial bioenergetics and secondly, the antioxidant capacity of these compounds on isolated liver mitochondria under severe oxidative damage conditions. The effect of these compounds was also tested on both the inner mitochondrial membrane anion channel (IMAC), which mediates the electrophoretic transport of a wide variety of anions and is believed to be an important component of the volume homeostatic mechanism (Beavis, 1992) and on the inner membrane permeability transition pore (PTP), which is induced under high matrix Ca^{2+} loads, oxidative stress, and depolarization and is believed to contribute to cellular injury by releasing cytochrome *c* and other factors able to trigger apoptosis (O'Rourke, 2000). The interaction of these four DHP derivatives with these channels could be relevant to the preservation of the mitochondrial structure and function and, consequently, to their potential protective action.

2. Materials and methods

2.1. Animals

Male Wistar rats (250–350 g), housed at 22 ± 2 °C under artificial light for 12-h light/dark cycle and with access to water and food ad labium, were used throughout the experiments. The experiments reported here were carried out in accordance with the National Requirements for Vertebrate Animal Research and the European Convention for the Protection of Animals used for Experimental and other Scientific Purposes.

2.2. Isolation of rat liver mitochondria

Rat liver mitochondria were isolated from male Wistar rats (6 weeks) by differential centrifugation according to conventional methods (Gazotti et al., 1979). After washing, the pellet was gently resuspended in the washing medium at a protein concentration of about 50 mg ml^{-1}. Protein content was determined by the biuret method (Gornall et al., 1949), using bovine serum albumin as standard.

2.3. Measurement of respiratory activities

Oxygen consumption of isolated mitochondria was

measured polarographically with a Clark oxygen electrode connected to a suitable recorder, in a 1 ml thermostated water-jacked, closed chamber with magnetic stirring at 30 °C. The respiratory reaction medium consisted of 250 mM sucrose, 10 mM HEPES pH 7.2, 20 mM KCl, 5 mM potassium phosphate, and 2 mM $MgCl_2$. Mitochondria (1 mg protein) were incubated in 1 ml reaction medium supplemented with 2 μM rotenone (in the presence and absence of compounds) for 3 min, before energization with 10 mM succinate. To induce state 3 respiration, adenosine diphosphate (ADP, 150 μM) was added. The respiratory control ratio (RCR) and ADP to oxygen ratio (ADP/O) were calculated according to the method previously described (Chance and Williams, 1956), considering that the saturation oxygen concentration was 232 nmol O_2/ml in the reaction medium at 30 °C. Uncoupled respiration was initiated by the addition of 1 μM *p*-trifluoromethoxyphenylhydrazone (FCCP).

2.4. Measurement of mitochondrial transmembrane potential ($\Delta\Psi$)

The mitochondrial transmembrane potential ($\Delta\Psi$) was measured indirectly based on the activity of the lipophilic cation tetraphenylphosphonium (TPP^+) using a TPP^+-selective electrode in combination with a Ag/AgCl-saturated reference electrode as previously described (Kamo et al., 1979). Both the TPP^+ and the reference electrode were inserted into an open glass chamber equipped with magnetic stirring and a thermostat set at 30 °C, and were connected to a pH meter. The signal was fed to a dual-trace potentiometric recorder. Mitochondria (1 mg) were incubated in 1 ml of respiration reaction medium supplemented with 2 μM rotenone and 3 μM TPP^+ (in the presence or absence of the compounds) for 3 min, before energization with 10 mM succinate. The $\Delta\Psi$ was estimated as previously indicated (Kamo et al., 1979) from the following equation: $\Delta\Psi = 59 \times \log(v/V) - 59 \times \log(10^{\Delta E/59} - 1)$ where v, V, and ΔE stand for mitochondria volume, volume of incubation medium, and deflection of the electrode potential from the baseline, respectively. A matrix volume of 1.1 μl/mg of protein was assumed. No correction was made for '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. As a consequence, we can anticipate some overestimation for the $\Delta\Psi$ values. Cerebrocrast, gammapyrone, glutapyrone, and diethone did not affect TPP^+ binding to mitochondria membranes or the electrode response.

2.5. Mitochondrial swelling

Mitochondrial osmotic volume changes were measured by the apparent absorbance changes at 520 nm with a suitable spectrophotometer-recorder set up. The reactions were carried out at 30 °C in 2.5 ml of the required media as indicated in the legends to figures.

2.6. Evaluation of mitochondrial membranes oxidative damage induced by ADP/Fe^{2+}

Mitochondrial membranes oxidative damage (lipid peroxidation) was induced by using ADP and iron (Fe^{2+}, $FeSO_4$) as oxidizing agents. Mitochondria (1 mg) were pre-incubated for 3 min at 30 °C in 1 ml of a standard medium containing 175 mM KCl, 10 mM Tris–Cl pH 7.4 supplemented with 3 μM rotenone (in the presence or absence of compounds) to avoid mitochondrial respiration induced by endogenous respiratory substrates. Membrane lipid peroxidation was induced by adding simultaneously 1 mM ADP and 0.1 mM $FeSO_4$ (ADP/Fe^{2+}). Iron solution was prepared immediately before use and protected from light. Controls were incubated at 30 °C during the same period of time, in the absence of ADP/Fe^{2+}, with or without the compounds.

Membrane lipid peroxidation was evaluated by measuring two different parameters, oxygen consumption, and thiobarbituric acid reactive substances (TBARS) formation (Ernster and Nordenbrand, 1967). For the TBARS assay, 0.5 ml aliquots of mitochondria suspensions, removed 10 min after the addition of ADP/Fe^{2+} were added to 0.5 ml of ice cold 40% trichloroacetic acid. Then, 2 ml of 0.67% of aqueous thiobarbituric acid containing 0.01% of 2,6-di-*tert*-butyl-*p*-cresol was added. The mixtures were heated at 90 °C for 15 min, cooled in ice for 10 min, and centrifuged at 850 *g* for 10 min. The supernatant fractions were collected and lipid peroxidation was estimated spectrophotometrically at

530 nm. The amount of TBARS formed was calculated using a molar extinction coefficient of $1.56 \times 105\ mol^{-1}\ cm^{-1}$ and expressed as nmol TBARS/mg protein (Buege and Aust, 1978). When the effect of diethone on $\Delta\Psi$ dissipation induced by lipid peroxidation was evaluated, the standard medium was supplemented with 3 μM rotenone and 1 μg oligomycin and mitochondria were energized with 10 mM succinate before initiation of lipid peroxidation by the addition of ADP/Fe^{2+}.

2.7. Statistical analysis

All the experiments were performed using at least four to six independent experiments with different mitochondrial preparations. The values are expressed as means ± S.E. Means were compared using ANOVA, post-test Student–Newmann–Keuls. Statistical significance was set at $p < 0.05$.

2.8. Chemicals

All chemicals were obtained from Sigma, St. Louis, MO, USA, except cerebrocrast, gammapyrone, glutapyrone, and diethone which were synthesized at the Latvian Institute of Organic Synthesis, 21 Aizkraukles Street, Riga, LV-1006, Latvia. Gammapyrone and glutapyrone were dissolved in water. Cerebrocrast and diethone were dissolved in absolute dimethyl sulfoxide (DMSO). Pure solutions of DMSO were added to controls with the highest volume used of both compounds' DMSO solutions [0.1% (v/v) of the experiments final volume] and had no effects on the measured activities.

3. Results

The effects of cerebrocrast, gammapyrone, glutapyrone, and diethone on succinate-dependent respiratory indexes, RCR, and ADP/O ratio of rat liver mitochondria are shown in Fig. 2. Glutapyrone, gammapyrone, and diethone concentration up to 100 μM, as well as cerebrocrast in the concentration range up to 25 μM, do not significantly affect the RCR and ADP/O. For a higher concentration range (up to 100 μM), cerebrocrast significantly depresses RCR and ADP/O ratio.

Fig. 3 shows the effects of cerebrocrast, gammapyrone, glutapyrone, and diethone on the respiratory rates characteristic of state 4 (succinate alone), FCCP-stimulated respiration (uncoupled respiration), and ADP-stimulated respiration (state 3) of rat mitochondria. State 3 and uncoupled respiration rates were significantly depressed by cerebrocrast for concentrations up to 100 μM, whereas state 4 respiration was stimulated three times. Gammapyrone, glutapyrone, and diethone concentrations up to 100 μM were without significant effect on the respiratory rates.

Fig. 4 shows the effect of cerebrocrast on mitochondrial transmembrane potential ($\Delta\Psi$) and phosphorylation rate developed by succinate-supported respiration. Cerebrocrast, in the concentration range up to 25 μM, does not significantly affect $\Delta\Psi$ developed by succinate-supported respiration. Concomitantly, the ADP phosphorylation rate was not

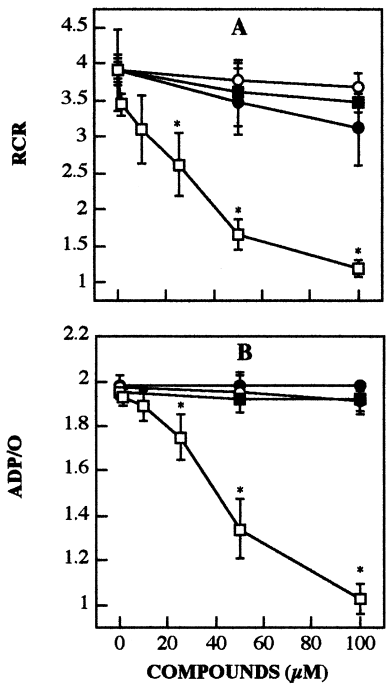

Fig. 2. Effects of cerebrocrast (□), glutapyrone (•), gammapyrone (○), and diethone (■) on succinate-dependent respiratory indexes RCR (A) and ADP/O (B) of rat liver mitochondria. Control values for rat liver mitochondria RCR = 3.9 ± 0.56; ADP/O ratio = 1.98 ± 0.05. The results correspond to the mean ± S.E. of four to six independent experiments. *$p < 0.05$ compared with the control (in the absence of compounds).

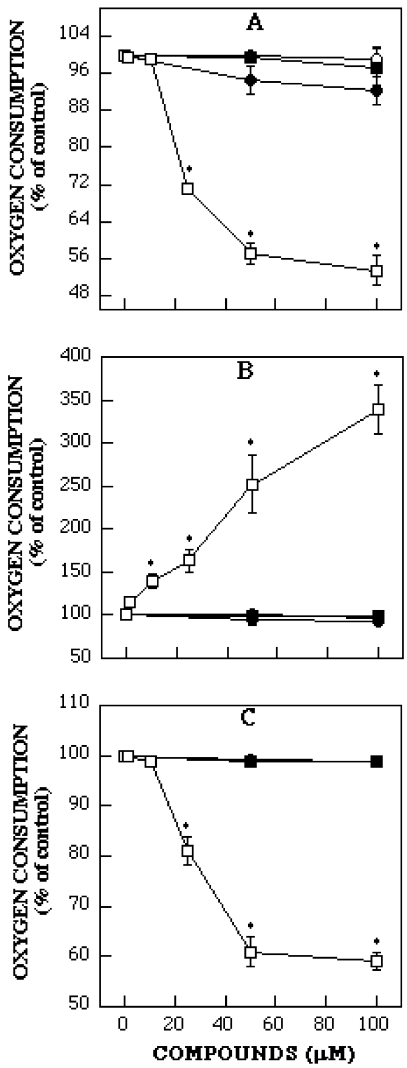

Fig. 3. Effects of cerebrocrast (□), glutapyrone (•), gammapyrone (○), and diethone (■) on succinate-supported O_2 consumption in state 3 (A), state 4 (B), and uncoupled-respiration (C) of rat liver mitochondria (when the symbols are not visible, they are overlapped). The following are the control values for rat liver mitochondria: state 3 = 68.35 ± 9.72; state 4 = 13.6 ± 2.9; uncoupled respiration = 83.83 ± 10.11, expressed in nmol O_2 (mg protein)$^{-1}$ min^{-1}. The results correspond to the mean ± S.E. of four independent experiments (when the error bars are not visible, S.E. is encompassed by the size of the symbols). *$p < 0.05$ compared with the control (in the absence of compounds).

changed. At a higher concentration range (up to 100 μM), it induces a $\Delta\Psi$ dissipation that affects the ADP phosphorylation rate. Partial dissipation of mitochondrial $\Delta\Psi$, by the action of the classical protonophore dinitrophenol (DNP, 10 μM) to values lower than those induced by cerebrocrast, had a comparatively smaller effect on the ADP phosphorylation rate (Fig. 4), suggesting that cerebrocrast-induced decrease of phosphorylation rate is related to phenomena in addition to $\Delta\Psi$ dissipation, i.e. concerning the phosphorylation system (phosphate carrier, ATP-synthase complex, and/or ATP/ADP carrier). Gammapyrone, glutapyrone, and diethone, in the concentration range up to 100 μM, do not significantly affect either $\Delta\Psi$ or phosphorylation rate developed by succinate-supported respiration.

Fig. 5 shows the effect of cerebrocrast on the mitochondrial phosphate carrier (phosphate$^-$/H^+ symporter), as evaluated by the passive swelling of rat liver mitochondria suspended in ammonium phosphate medium. In the absence of cerebrocrast, non-respiring mitochondria, suspended in ammonium phosphate medium swell, spontaneously due to phosphate enter in symport with H^+. In the presence of cerebrocrast concentrations up to 50 μM, the rate of mitochondrial swelling decreased in a concentration-dependent manner. This finding indicates that cerebrocrast causes a partial inhibition of phosphate transport and, consequently, depression on the phosphorylation rate. However, the inhibitory action of cerebrocrast on the phosphate carrier per se did not explain the depression on the phosphorylation rate observed for concentrations above 50 μM (Fig. 4). In this concentration range, the effect of cerebrocrast on the rate of mitochondrial swelling was identical (Fig. 5). The effect of cerebrocrast on the phosphate carrier was confirmed by swelling induced by activation of the phosphate and dicarboxylate carriers, i.e. by the addition of 5 mM phosphate to non-respiring mitochondria suspended in potassium malate medium plus nigericin, as described by Zernig et al. (1990) (results not shown). Depression of the phosphorylation rate induced by cerebrocrast in rat liver mitochondria cannot be attributed to the inhibition of the ATP-synthase, since the activity of mitochondrial ATPase was not significantly affected by 100 μM cerebrocrast (results not shown). The effect of cerebrocrast on the ATP/ADP carrier was not evaluated and its involvement in the depression of the phosphorylation rate induced by this compound cannot be excluded.

In Fig. 6, we can observe the effect of cerebrocrast

Cerebrocrast (μM)	ΔΨ (mV)		
	Succinate energization	ADP depolarization	Repolarization
0	215 ± 0.7	14 ± 0.8	214 ± 0.6
25	211 ± 2.8	13 ± 0.8	210 ± 2.7
50	208 ± 3.4	12 ± 0.6	206 ± 3.5
75	206 ± 3.9	9 ± 0.4*	202 ± 3.0*
100	198 ± 4.0*	8 ± 0.2*	196 ± 3.7*

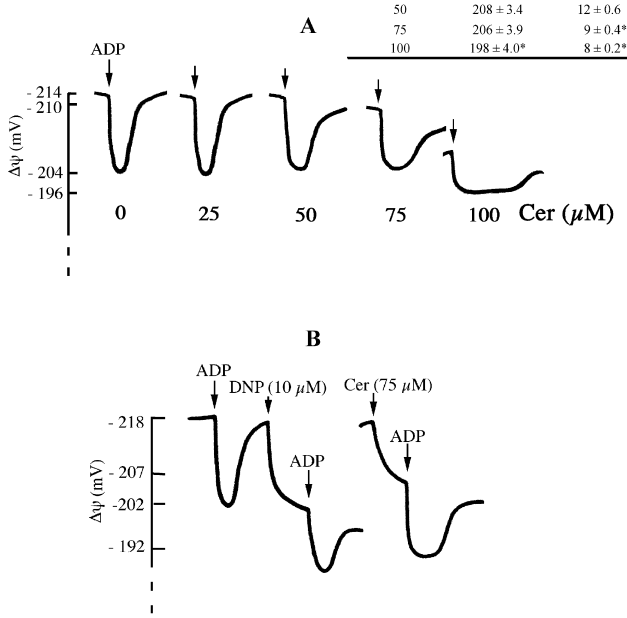

Fig. 4. Effect of cerebrocrast on mitochondrial transmembrane potential (ΔΨ) and phosphorylation rate of rat liver mitochondria supported by succinate (A) and mitochondrial phosphorylation after partial ΔΨ dissipation by dinitrophenol (DNP) as a reference, for the evaluation of cerebrocrast effects (B). Mitochondria (1 mg) were incubated in 1 ml of the standard respiratory medium supplemented with 2 μM rotenone and 3 μM TPP^+ for 3 min at 30 °C in the absence and presence of cerebrocrast (25, 50, 75, and 100 μM), before energization with 10 mM succinate. The phosphorylation was initiated with the addition of ADP (150 μM). Cer indicates addition of cerebrocrast and DNP, addition of dinitrophenol. The traces are typical of several independent experiments with four different mitochondrial preparations. Table inserts show the average response (mean ± S.E.) of membrane potential developed with succinate, the drop in membrane potential after ADP addition, and the repolarization value after all the added ADP were phosphorylated. Values statistically different from control (in the absence of cerebrocrast): $^*p < 0.05$.

on the IMAC, as evaluated by the passive swelling of rat liver mitochondria suspended in KCl medium, after activation of IMAC by depleting matrix divalent cations with the ionophore A23187 (Beavis, 1992). In the absence of cerebrocrast, non-respiring mitochondria suspended in KCl medium swells very slowly. The addition of A23187 depletes the matrix of divalent cations and in the presence of nigericin, to induce the K^+/H^+ antiport, a small increase in mitochondrial volume, associated with the net exchange of osmotically inactive Mg^{2+} for osmotically active K^+, indicates the activation of a Cl^- channel. A rapid swelling is not observed until valinomycin is added to provide a uniport pathway for K^+. Thus, in the presence of sufficient valinomycin to ensure that K^+ transport is not limiting, the rate of KCl influx and subsequent swelling should be limited by the rate of Cl^- entry into mitochondrial matrix via IMAC. Treatment of mitochondria with cerebrocrast concentrations up to 25 μM inhibited this type of swelling in a concentration-dependent manner indicating that Cl^- transport has been inhibited. The IC_{50} value, concentration causing 50% of maximal inhibition, calculated as previously described (Zernig et al., 1990) for IMAC inhibition by cerebrocrast was 10.5 ± 2.0 μM. Inhibition of phosphate uniport by cerebrocrast was demonstrated, by a similar procedure in a potassium phosphate medium using mitochondria treated with *N*-ethylmaleimide to block the electroneutral transport of phosphate by the $phosphate^-/H^+$ symporter

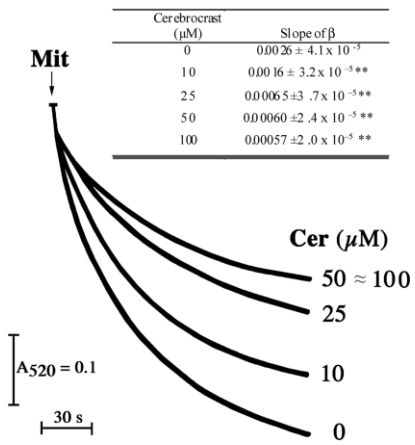

Fig. 5. Effect of cerebrocrast on the phosphate carrier, evaluated by passive swelling of rat liver mitochondria suspended in ammonium phosphate medium. Mitochondria (1 mg) were suspended in 2.5 ml of ammonium phosphate medium [135 mM ammonium phosphate, 5 mM HEPES (pH 7.2) and 0.1 mM EDTA] supplemented with 2 μM rotenone at 30 °C in the absence and presence of cerebrocrast (10, 25, 50, and 100 μM). Mit indicates the addition of mitochondria and Cer, addition of cerebrocrast. The traces are typical of several independent experiments with four different mitochondrial preparations. Table inserts show the average response (mean ± S.E.) of the slope of β for the first 60 s. β is a light scattering variable which normalizes reciprocal absorbance (A^{-1}) for mitochondrial concentration, P (mg/ml). $\beta \equiv P/P_s(A^{-1} - a)$ where P (equals 1 mg/ml) is introduced to make β a scaled dimensionless quantity and a is a machine constant equal to 0.05 with our apparatus (Beavis and Garlid, 1987). Values statistically different from control (in the absence of cerebrocrast): $^{**}p < 0.005$.

(results not shown). In the same concentration range, gammapyrone, glutapyrone, and diethone were without effect on both mitochondrial Cl^- and phosphate uniporters.

The effect of cerebrocrast on Ca^{2+}-induced opening of mitochondrial PTP was evaluated by following mitochondrial swelling (Fig. 7). Mitochondria have a finite capacity to accumulate calcium before undergoing the Ca^{2+}-dependent mitochondrial permeability transition (Rolo et al., 2000). The Ca^{2+} concentration that should be applied to assess the effect of cerebrocrast on PTP induction was evaluated and a Ca^{2+} concentration of 40 μM was selected. In the absence of cerebrocrast, Ca^{2+}-loaded, succinate-energized rat liver mitochondria suspension underwent a permeability transition, as reflected by the decrease in absorbance at 520 nm, an effect that was inhibited completely by prior addition of Cyscloporine (CsA). Treatment of mitochondria with cerebrocrast concentrations up to 25 μM enlarged the lag time required to exert their effects, but the rate of swelling was not altered as compared with control mitochondria. However, the magnitude of the volume change of mitochondria treated with cerebrocrast decreased in a concentration-dependent manner. This finding indicated that the induction of mitochondrial swelling was not completed and the effect of cerebrocrast was not only due to the alteration of the time-constant governing activation of PTP. Cerebrocrast concentrations higher than 25 μM did not significantly protect Ca^{2+}-induced mitochondrial swelling (results not shown). This finding is not surprising if we take into account that cerebrocrast concentrations

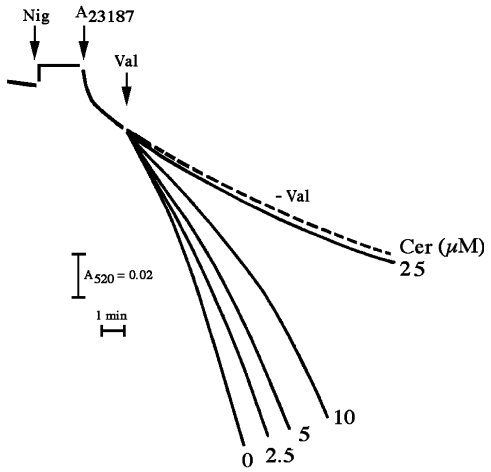

Fig. 6. Effect of cerebrocrast on the mitochondrial inner membrane channel (IMAC), evaluated by passive swelling of rat liver mitochondria suspended in KCl medium. Mitochondria (1 mg) suspended in 2.5 ml of KCl medium [54 mM KCl, 5 mM HEPES (pH 7.4), 0.1 mM EGTA, and 0.2 mM EDTA] supplemented with 2 μM rotenone were incubated for 3 min at 30 °C in the absence and presence of cerebrocrast (2.5, 5, 10, and 25 μM). At the end of the incubation period, nigericin (1 nmol/mg protein) was added followed by the addition of A23187 (10 nmol/mg protein) and valinomycin (1 nmol/mg protein) 2 and 4 min later, respectively. In all the assays, nigericin and A23187 were present; (—) assays performed in the presence of valinomycin and (- - - - -) assay performed in the absence of valinomycin and cerebrocrast. Nig, A23187, and Val, indicate the additions of nigericin, A23187, and valinomycin, respectively. The traces are typical of several independent experiments with four different mitochondrial preparations.

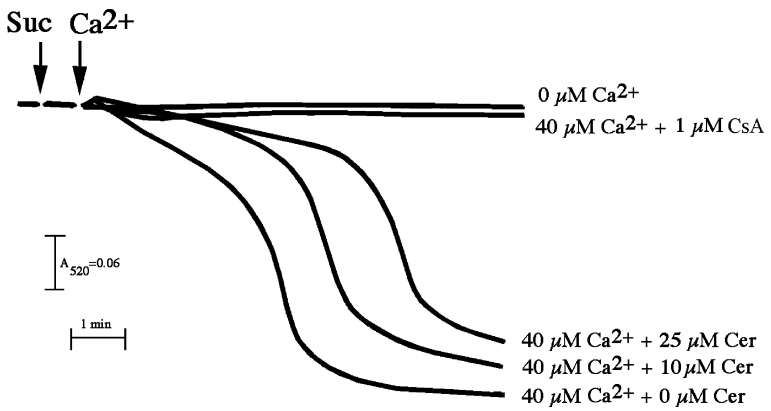

Fig. 7. Effect of cerebrocrast on Ca^{2+}-induced opening of mitochondrial PTP, evaluated by passive mitochondrial osmotic swelling. Mitochondria (1 mg) suspended in 2.5 ml of an osmotic medium containing 200 mM sucrose, 10 mM HEPES–Tris (pH 7.4), 1 mM KH_2PO_4, and 10 μM EGTA supplemented with 2 μM rotenone and 0.5 μg/ml oligomycin were incubated for 3 min at 30 °C in the absence and presence of cerebrocrast (10 and 25 μM). At the end of the incubation period, 5 mM succinate was added and the experiments were started by the addition of 40 μM Ca^{2+}, 2 min later. When indicated, 1 μM Cyclosporine A (CsA) was included in the reaction medium prior the addition of succinate and Ca^{2+}. The traces are typical of several independent experiments with four different mitochondrial preparations.

higher than 25 μM cause mitochondrial uncoupling (Figs. 2 and 4), an effect that potentiates PTP by itself. Although the uncoupling action of high concentrations of cerebrocrast (≥25 μM) likely limits its potential protective effect in vivo, the partial protective action of low concentrations of cerebrocrast (≤25 μM) observed in rat liver mitochondria (Fig. 7) could be related to the neuroprotective action of low doses of cerebrocrast administered to rats and mice in vivo (0.005–0.05 mg kg^{-1} i.p., i.v., and p.o.) (Klusa, 1995). Gammapyrone, glutapyrone, and diethone were without effect on Ca^{2+}-induced PTP.

Fig. 8 shows the effect of different concentrations of diethone on oxygen consumption (A), TBARS formation (B), and ΔΨ dissipation (C) due to lipid peroxidation initiated by the oxidant pair ADP/Fe^{2+} in rat liver mitochondria. In the absence of diethone and after the addition of ADP/Fe^{2+}, it is possible to distinguish a two-phase kinetics in oxygen consumption: an initial, slow oxygen consumption lag phase lasting about 1 min is followed by a rapid oxygen consumption phase. The lag phase is considered to be the time required for the generation of a sufficient amount of the perferryl ion complex (ADP–Fe^{2+}–O_2 ↔ ADP–Fe^{3+}–O_2^-) which has been suggested to be responsible for the initiation of lipid peroxidation. The rapid oxygen consumption phase is probably due to the oxidation of the polyunsaturated fatty acid acyl chain of membrane phospholipids by ROS and, consequently, to the propagation phase of lipid peroxidation (Sassa et al., 1990). Diethone concentrations up to 5 μM enlarge the slow oxygen consumption lag phase/lag time, before oxygen uptake burst induced by the complex ADP/Fe^{2+}, but were without significant effect on the rapid oxygen consumption phase. At 5 μM diethone, a complete lipid peroxidation inhibition is observed.

The quantitative evaluation of TBARS formation, induced by ADP/Fe^{2+}, was performed to confirm the clear antioxidant effect of diethone also observed by oxygen consumption. Fig. 8B shows that the kinetics of TBARS formation induced by ADP/Fe^{2+} is similar to that observed for oxygen consumption. TBARS formation was inhibited in the same range of diethone concentrations observed to inhibit oxygen consumption.

The effect of diethone on ΔΨ dissipation, due to liver mitochondrial membranes lipid peroxidation, initiated by ADP/Fe^{2+} after mitochondrial energization with succinate, was also evaluated (Fig. 8C). The energization of mitochondria was carried out in the presence of oligomycin, a specific inhibitor of mitochondrial ATP synthase to avoid the membrane depolarization that follows ADP/Fe^{2+} addition. Upon succinate energization and in the absence of ADP/Fe^{2+}, mitochondria built up and sustained a ΔΨ close

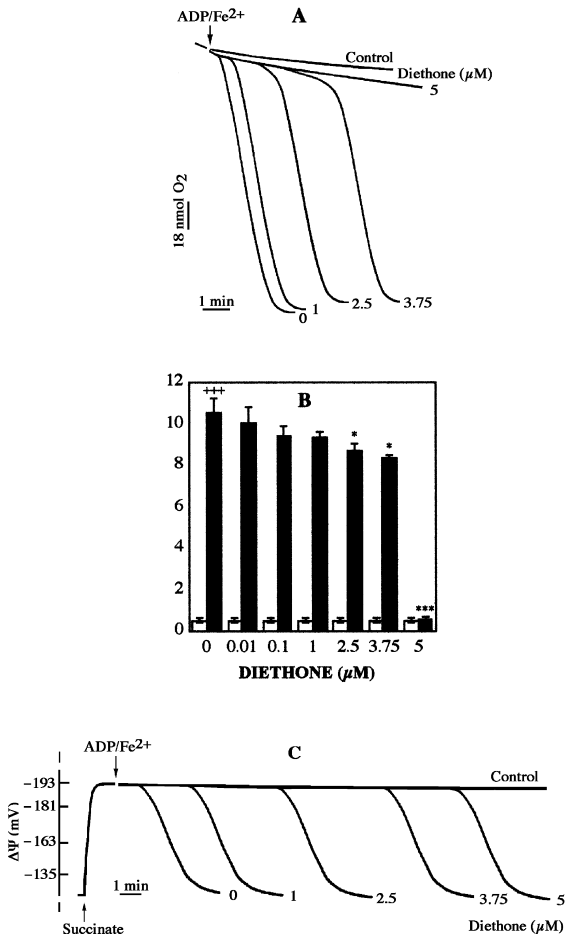

Fig. 8. Effect of diethone on mitochondrial oxidative damage (lipid peroxidation) induced by the oxidant pair ADP/Fe^{2+}. Lipid peroxidation was evaluated by oxygen consumption (A), TBARS assay (B), and $\Delta\Psi$ (C). The traces in (A) and (B) represent typical direct recordings representative of four to six independent experiments with different mitochondrial preparations. The data in (B) correspond to the mean ± S.E. of four to six independent experiments. $^{*}p < 0.05$, $^{***}p < 0.0001$ compared with mitochondria submitted to ADP/Fe^{2+} treatment (B) in the absence of diethone. $^{+++}p < 0.0001$ compared with mitochondria not submitted to ADP/Fe^{2+} treatment (G).

to − 200 mV for more than 15 min. After the addition of ADP/Fe^{2+}, a lag phase was observed that, similar to what happened with oxygen consumption, represents the time required to generate a sufficient amount of ROS responsible for the initiation of lipid peroxidation. After this lag phase, the $\Delta\Psi$ underwent a rapid decline, probably associated with the disruption of mitochondrial membrane promoted by lipid peroxidation. Diethone increased the depolarization lag phase in a dose-dependent manner, but it was without effect on the rapid decline of the $\Delta\Psi$. It is important to note that maximum protection with diethone on $\Delta\Psi$ was attained in the concentration range up to 5 μM, where it shows a maximum antioxidant capability, as determined by the quantification of TBARS and oxygen consumption. Cerebrocrast, gammapyrone, and glutapyrone concentrations up to 100 μM did not affect ADP/Fe^{2+}-induced lipid peroxidation of rat liver mitochondria, as confirmed by the parameters used to evaluate lipid peroxidation.

4. Discussion

In this study, we investigated whether four structurally different DHP derivatives (cerebrocrast, gammapyrone, glutapyrone, and diethone), at the concentration range showing no interference with mitochondrial bioenergetics, can protect mitochondria against oxidative stress.

One of the main findings was the observation that, from the four compounds tested, cerebrocrast is the only compound that affects mitochondrial bioenergetics, but only at concentrations higher than 25 μM, as shown by the following parameters: decrease of the succinate-dependent respiratory indexes (RCR and ADP/O) (Fig. 2); inhibition of oxygen-supported respiration in state 3 and uncoupled respiration (Fig. 3) with parallel $\Delta\Psi$ dissipation (Fig. 4) and decrease of the phosphorylation efficiency (Fig. 4); inhibition of the phosphate carrier (Fig. 5) and stimulation of oxygen-supported respiration in state 4 (Fig. 4). These data suggested that cerebrocrast at high concentrations (≥25 μM) exhibited a toxic action. The toxic action of cerebrocrast, as evaluated by its interference with mitochondrial bioenergetics, fits well with toxicity evaluated by its LD_{50} values 450 (357–567) mg kg^{-1} i.p. and 730 (570–934) mg kg^{-1} p.o. in mice (Misāne et al., 1993).

Other main finding was the observation that cerebrocrast, at concentrations that did not interfere with mitochondrial bioenergetics (≤25 μM), was also the only compound that had the capacity to inhibit IMAC (Fig. 6). This effect of cerebrocrast is not surprising if we take into account that its structure is more related to the Ca^{2+} antagonist structure of

DHPs class, well known inhibitors of IMAC (Zernig et al., 1990) than to the other three compounds (Fig. 1). Also, the IC_{50} value for IMAC inhibition by cerebrocrast ($IC_{50} = 10.5 \pm 2.0$) is similar to those reported for nicardipine ($IC_{50} = 8.4 \pm 0.4$), niludipine ($IC_{50} = 8.7 \pm 0.8$), and nisoldipine ($IC_{50} = 12 \pm 2.7$), well known Ca^{2+} antagonist of DHPs class (Zernig et al., 1990). IMAC, which is inhibited by matrix Mg^{2+} and H^+, only appears to conduct ions under low divalent ions matrix concentrations and alkaline matrix conditions, being unclear what its physiological role is or if it opens under pathophysiological conditions (O'Rourke, 2000). However, it has been suggested to be an important safeguard against mitochondrial swelling, as it is poised to counteract the influx of cations (Beavis, 1992). Accordingly, Zernig et al. (1990) proposed that the inhibition of IMAC by Ca^{2+} antagonists (DHPs, phenylalkylamines, and benzothiazepines) could inhibit excessive Ca^{2+} uptake by mitochondria, preventing the resulting structural and functional impairment. In agreement with this proposition, the partial protective action of low concentrations of cerebrocrast (≤ 25 μM) on Ca^{2+}-induced PTP (Fig. 7) may be a consequence of its inhibitory action on the transport of phosphate via IMAC (results not shown), thus, preventing excessive Ca^{2+} accumulation inside mitochondria and, consequently, decreasing their susceptibility to PTP induction. The importance of the interaction of cerebrocrast with IMAC is supported by a recent suggestion that some of the effects of Ca^{2+} antagonists, including the neuroprotective and anticonvulsant effects of DHPs (nifedipine, nitrendipine, nicardipine, and (R)-Bay K 8644), might result partly from their interactions with ligand-gated chloride channels (Chesnoy-Marchais and Cathala, 2001) and also by the finding showing that inhibitors of IMAC (e.g. amiodarone, amitriptyline, tributyltin, propanolol, and PK11195, a peripheral benzodiazepine receptor inhibitor) are able to suppress the mitochondrial oscillations namely, periodic rapid oxidation of mitochondrial flavoproteins and the collapse of $\Delta\Psi$, induced in intact cardiomyocytes under metabolic stress (O'Rourke, 2000). Metabolic stress causes oxidative stress in human breast carcinoma MCF-7/ADR cells (Lee et al., 1998), an effect that has been suggested to be related to ROS production by mitochondria during glucose deprivation (Spitz et al., 2000; Lee et al., 2001). Therefore, inhibitors of IMAC, including cerebrocrast, may be of great value in the treatment of several pathological situations known to be associated with cellular oxidative stress.

Another point of interest was the finding that gammapyrone, glutapyrone, and cerebrocrast did not protect rat liver mitochondria against oxidative damage induced by the oxidant pair ADP/Fe^{2+}, while diethone could protect in a dose-dependent manner (Fig. 8). This effect is probably due to the prevention of the formation of ROS derived from ADP/Fe^{2+}, namely, the perferryl ion complex ADP–Fe^{3+}–O_2^-, which has been suggested to be responsible for the initiation phase of lipid peroxidation, analyzed as the lag time that accompanies the oxygen uptake burst and the collapse of $\Delta\Psi$ (Fig. 8). Mitochondrial membranes were used to demonstrate the antioxidant activity of diethone because they are particularly susceptible to oxidative damage due to its high content in polyunsaturated fatty acids (Halliwell and Gutteridge, 1999; Kowaltowski and Vercesi, 1999) and proximity to the ROS generated in the electron transport chain (Lee and Wei, 2000; Murphy and Smith, 2000; Pedersen, 2000). Mitochondrial oxidative damage causes alterations in structural integrity of mitochondrial membranes that lead to $\Delta\Psi$ dissipation, depletion of cellular ATP, and cell death (Kowaltowski and Vercesi, 1999). Previous studies demonstrated that preservation of mitochondrial $\Delta\Psi$ is critical to maintain cell integrity (Carini et al., 1990). Therefore, diethone, by preventing the disruption of mitochondrial $\Delta\Psi$ that follows peroxidative alterations, might protect mitochondrial function thus preventing irreversible cell injury in pathological situations associated with cellular oxidative stress. The results obtained in this study are quite consistent with previous findings showing that diethone, under the same concentration range, exhibits antioxidant properties in rat liver mitochondria, expressed as the enlargement of the induction period/lag time before mitochondrial swelling and oxygen uptake burst induced by the complex ADP/Fe^{2+} in the presence of ascorbate (reducing equivalent), which corresponds to the inhibition of the initiation of lipid peroxidation (Velena et al., 1999). At small concentrations (comparable with pharmacological active ones), diethone has been shown to inhibit Fe^{2+}

initiated lipid peroxidation, not only in mitochondria but also in isolated rat liver microsomes. In phosphatidylcholine liposomes, when lipid peroxidation was initiated by methemoglobine and in emulsions of linethole, if this initiation was made in the presence of the heme compounds hemine, hematine, and cytochrome *c*, the antioxidant effect of diethone was also observed (Abdalla et al., 1999; Velena et al., 1999).

The comparison of the effects of cerebrocrast, gammapyrone, glutapyrone, and diethone on mitochondria bioenergetics, IMAC, Ca^{2+}-induced opening of PTP, and oxidative damage induced by ADP/Fe^{2+}, clearly showed that none of the compounds interfere with mitochondrial bioenergetics at concentrations lower than 25 μM; cerebrocrast was the only compound that affected mitochondrial bioenergetics, but only for concentrations higher than 25 μM; at concentrations that did not affect mitochondrial bioenergetics (≤ 25 μM), only cerebrocrast inhibited the IMAC and partially prevented Ca^{2+}-induced opening of the PTP; diethone was the only compound that expressed antioxidant activity at very low concentrations (≤ 5 μM). The different effects of cerebrocrast and diethone cannot be attributed to differences on their lipid solubility since they are both lipophilic compounds (Misãne et al., 1993; Klusa, 1995; Velena et al., 1997, 1999). The apparent partition coefficient of cerebrocrast, log $P = 4.96$ (octanol/water) (Tirzite, personal communication) and that of diethone, log $P > 2$ (linethole/phosphate buffer pH 7.5) (Kumsars et al., 1971) favor the presence of these molecules in the bulk phase of biological membranes. The different effects of cerebrocrast and diethone on mitochondria might be related with differences on their molecular structure (Fig. 1). The interference of cerebrocrast with mitochondrial function may be related with the 2-difluoro-methoxyphenyl ring, which is joined to DHP at position 4 in combination with the medium length 2-propoxyethyl side chains with oxa atoms in positions 3 and 5 of the DHP ring. The potent antioxidant capacity of diethone is probably related to the non-substituted methylene group, having two hydrogen atoms in position 4 of the DHP molecule, capable of being proton donors.

In conclusion, in this study, we found that among the DHP derivatives (cerebrocrast, gammapyrone, glutapyrone, and diethone) examined, cerebrocrast acting as an inhibitor of IMAC and diethone, acting as an antioxidant, could provide effective protective roles in preventing mitochondria from oxidative damage, favoring their therapeutic interest in the treatment of several pathological situations known to be associated with cellular oxidative stress. Studies using whole animals or organs (i.e. in vivo or ex vivo organ function studies) and cultured cells (i.e. in vitro energy metabolism studies), as well as studies with other potent lipophilic antioxidants, deserve further investigation to validate the relevance of the effects of cerebrocrast and diethone on isolated mitochondria function.

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