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Targeting the Mitochondria by Novel Adamantane-Containing 1,4-Dihydropyridine Compounds

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1. Introduction

Mitochondria are important regulators of cellular functions and energy metabolism, therefore mitochondrial dysfunction leads to a compromised energy-generating system, deteriorated cellular homeostasis and neurodegenerative disorders, such as Parkinson's disease and Alzheimer's disease (Shapira, 1999; 2009). Hence, the protection of mitochondria, even their repair mechanisms at the level of complex I, may be a key strategy in limiting mitochondrial damage and ensuring cellular integrity (Dawson & Dawson, 2003). Thus, in addition to traditionally used antiparkinsonian drugs, which are focused on the activation of the dopaminergic system, different mitochondria-protecting agents are being used in clinics for the treatment of Parkinson's disease. For instance, agents with antioxidant properties, such as melatonin (Esposito & Cuzzocrea, 2010), coenzyme Q10 and creatine (Kones, 2010), lipoic acid (De Araújo et al., 2011), and the extract of Hyoscyamus niger seeds (Sengupta et al., 2011), are currently used to treat Parkinson's disease.

Recently, antihypertensive drugs of the calcium antagonistic series, which belong to 1,4-dihydropyridine (DHP) class and are capable of penetrating the blood-brain barrier (e.g., nifedipine, nimodipine), were shown to significantly reduce the risk of developing Parkinson's disease (Becker et al., 2008; Ritz et al., 2010). This was explained by blocking L-type calcium channels in the dopaminergic neurons of the substantia nigra, where elevated calcium ion concentrations initiate cell death (Sulzeret & Schmitz, 2007). However, the mechanism of the antiparkinsonian action of DHPs is not yet understood.

Our investigation of DHP compounds showed that many of them are capable of protecting mitochondrial processes (Fernandes et al., 2003, 2005, 2008, 2009). For instance, the most

active compound cerebrocrast {4-[2-(difluoromethoxy)phenyl-2,6-dimethyl-1,4-dihydropiridine-3,5-dicarboxylic acid di(2-propoxyethyl)diester}, which has shown neuroprotective effects in different neurodeficiency models (Klusa, 1995), decreased mitochondrial toxin 1-methyl-4-phenylpyridinium (MPP^+)-induced cell death in rat cerebellar granule cells (Klimaviciusa et al., 2007). In isolated mitochondria of rat liver, cerebrocrast inhibited the inner mitochondrial anion channel, Ca^{2+}-induced opening of the mitochondrial membrane permeability transition pore and permeabilization of the mitochondrial inner membrane (Vicente et al., 2006). In addition, it normalized oxidative phosphorylation and increased adenosine triphosphate (ATP)-induced contraction in swollen mitochondria of isolated rat skeletal muscle (Velena et al., 1997). Cerebrocrast and its congeners also protected against histopathological changes caused by azidothymidine, known to be a mitochondrial toxin (Pupure et al., 2008).

The present study investigates two novel DHP compounds, cerebrocrast analogues containing structure elements that may enhance the delivery of molecules through the blood-brain barrier and improve their access to mitochondria. The compounds are composed of either one adamantane moiety in position 3 (AV-6-93) or two adamantane moieties in positions 3 and 5 (diflurone) of the DHP ring. We suggest that these DHP structures may possess mitochondria-protecting and antiparkinsonian activity due to both the adamantane moiety, which can be considered to be an important functional unit, and the DHP structure, which may serve as the carrier molecule. Adamantane molecules were previously used in the design of neuroprotective drugs. For example, amantadine (1-amino-adamantane) is used in antiparkinsonian drugs with mechanisms focused on NMDA-receptor gated ion channels (Kornhuber et al., 1991). Adamantane derivatives, particularly memantine, are reported as neuroprotective agents against mitochondrial toxicity *in vivo* (Rojas et al., 2008) and *in vitro* (McAllister et al., 2008). Memantine may act directly on dopamine D2High receptors (Seeman et al., 2008), whereas amantadine may stimulate the synthesis and release of dopamine in the rat striatum (Spilker & Dhasmana, 1973), which is beneficial in the treatment of Parkinson's disease. Aminoadamantane derivatives 4-(1-adamantylamino)-2,2,6,6-tetramethylpiperidine-1-oxyl and 4-(1-adamantylammonio)-1-hydroxy-2,2,6,6-tetramethylpiperidinium dihydrochloride were also synthesised as antiparkinsonian drugs (Skolimowski et al., 2003). However, compounds with adamantane moieties attached to the DHP structure have not yet been synthesised.

In this study, we tested novel compounds *in vitro* to assess their influence on mitochondrial processes in primary cultures of rat cortical neurons, using mitochondrial toxin MPP^+, and on isolated rat liver mitochondria.

2. Materials and methods

2.1 Animals

Male Wistar rats (250-350 g), housed at 22 ± 2 ^oC under artificial light for a 12-h light/dark cycle and with access to water and food *ad libitum*, were used for these experiments. All of the experimental procedures were performed in accordance with the guidelines of Directive 86/609/EEC "European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes" (1986) and were approved by the National Ethics Committee.

2.2 Chemicals

AV-6-93 [2,6- dimethyl-3-(1-adamantyloxycarbonyl)-4-(2-difluoromethoxyphenyl)-5-[(2-propoxy)ethoxycarbonyl]-1,4-dihydropyridine] (Fig. 1A) and diflurone [2,6- dimethyl-3,5-

bis(1-adamantyloxycarbonyl)-4-(2-difluoromethoxyphenyl)-1,4-dihydropyridine] (Fig. 1B) were synthesised at the Latvian Institute of Organic Synthesis, 21 Aizkraukles Street, Riga, LV-1006. AV-6-93 and diflurone were dissolved in 100% DMSO and further diluted to concentrations of 0.1% (v/v) and less.
Chemicals for the mitochondrial studies were obtained from Sigma Chemical Company (St Louis, MO, USA); chemicals for cytotoxicity studies mentioned in 2.3. and 2.4.

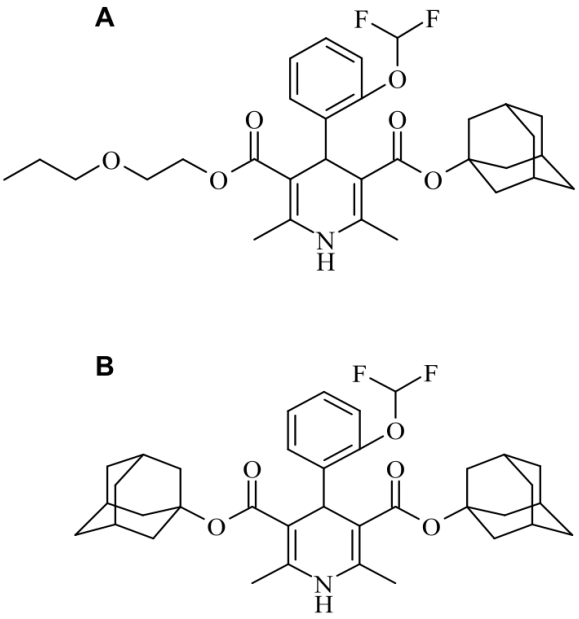

Fig. 1. The structures of AV-6-93 (A) and diflurone (B).

2.3 Primary culture of rat cortical neurons

Primary cultures were prepared from 1-day-old Wistar rat pups, according to the method of Alho et al., 1988, with minor modifications. Briefly, cortices were dissected in ice-cold Krebs-Ringer solution (135 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 0.4 mM KH_2PO_4, 15 mM glucose, 20 mM HEPES, pH 7.4, containing 0.3% bovine serum albumin) and trypsinised in 0.8% trypsin-EDTA (Invitrogen, U.K.) for 10 min at 37 °C, followed by trituration in 0.008% DNAse I solution containing 0.05% soybean trypsin inhibitor (both obtained from Surgitech AS, Estonia). Cells were resuspended in Eagle's basal medium with Earle's salts (BME, Invitrogen, U.K.), containing 10% heat-inactivated foetal bovine serum (FBS, Invitrogen, U.K.), 25 mM KCl, 2 mM GlutaMAX™-I (Invitrogen, U.K.) and 100 µg/mL gentamycin. Cells were plated onto poly-L-lysine- (Sigma Chemical Co., MO, USA) coated 48-well plates at a density of 1.8 x 10^5 cells/cm^2. The medium was changed to Neurobasal™-A medium containing 2 mM GlutaMAX™-I with B-27 supplement and 100 µg/mL gentamycin 2.5 hr later. Cultures were incubated for 6 days in a 5% CO_2/95% air atmosphere at 37 °C, and one-fifth of the culture medium was changed on DIV 3 (day 3 *in vitro*).

2.4 Measurement of cell death in cytotoxicity assay

Primary rat cortical neurons were cultured for 5 days as described above. On DIV 5, cultures were incubated with 1-methyl, 4-phenylpyridinium (MPP^+, Sigma Chemical Co., MO, USA) for the following 24 hr at a concentration of 300 μM. Cells were pre-incubated with the tested compounds AV-6-93 and diflurone for 90 min followed by the addition of MPP^+ and further incubation with MPP^+ plus the tested compounds or a solvent (control) for the next 24 hours. Cell death was measured with a Trypan blue assay (Tymianski et al., 1993). Cells were incubated with 0.4% Trypan blue solution in phosphate buffered saline (PBS, 145 mM NaCl, 3 mM KCl, 0.42 mM Na_2HPO_4, 2.4 mM KH_2PO_4, pH = 7.4) at 37 ^oC for 7 min and then washed twice with PBS and fixed with 4% paraformaldehyde in PBS. Only dead neurons were stained with Trypan blue (Tymianski et al., 1993). The fixed cultures were rinsed with PBS for microscopic observation, and approximately 150 cells per 5 fields in each well were counted to determine the number of dead cells and the total number of cells. Neuronal death was calculated as the percentage of dead cells from the total (viable plus dead) number of cells, and the obtained data were averaged for each well.

2.5 Isolation of rat liver mitochondria

Rat liver mitochondria were isolated from male Wistar rats 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. Protein content was determined by the biuret method (Gornall et al., 1949), using bovine serum albumin as a standard.

2.6 Measurement of respiratory activities

Oxygen consumption was monitored polarographically with a Clark-type electrode at 30 °C in a closed glass chamber equipped with magnetic stirring. Mitochondria (1 mg/ml) were incubated in a respiratory medium containing 130 mM sucrose, 5 mM HEPES (pH 7.2), 50 mM KCl, 2.5 mM K_2HPO_4, and 2.5 mM $MgCl_2$ (in the presence and absence of AV-6-93 or diflurone) for 3 min before energisation with 10 mM glutamate/5 mM malate. When 10 mM succinate was used as the respiratory substrate, the reaction medium was supplemented with 2 μM rotenone. To induce state 3 respiration, adenosine diphosphate (ADP, 150 μM) was added. FCCP (p-trifluoromethoxyphenylhydrazone)-stimulated respiration was initiated by the addition of 1μM FCCP. 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. The ADP/O ratio, which is expressed by the ratio between the amounts of ADP added and the oxygen consumed during state 3 respiration, is an index of oxidative phosphorylation efficiency. Respiration rates were calculated assuming that the saturation of oxygen concentration was 250 μM at 30 ^oC (Chance & Williams, 1956), and the values are expressed in percentage of control (% of control).

2.7 Measurement of mitochondrial transmembrane potential

The mitochondrial transmembrane potential ($\Delta\psi$) was measured indirectly based on the detection of lipophilic cation tetraphenylphosphonium (TPP^+) using a TPP^+-selective electrode, as previously described (Kamo et al., 1979). The $\Delta\psi$ was estimated from the following equation (1):

$$\Delta\psi = 59 \times \log (v/V) - 59 \times \log (10^{\Delta E/59} - 1) \quad (1)$$

where v, V, and ΔE stand for inner mitochondrial volume, incubation medium volume, and deflection of the electrode potential from the baseline, respectively. A mitochondrial matrix volume of 1.1 μl/mg protein was assumed. No correction was made for the "passive" binding of TPP^+ to the mitochondrial membranes because the purpose of the experiments was to show relative changes in potential rather than absolute values. As a consequence, we anticipate some overestimation for the Δψ values. To monitor Δψ associated with mitochondrial respiration, liver mitochondria (1 mg/ml) were incubated for 3 min in the respiratory medium described above, supplemented with 3 μM TPP^+, at 30 °C in the absence or presence of different concentrations of AV-6-93 or diflurone before energisation with 10 mM glutamate/5 mM malate or 10 mM succinate. When succinate was used as the respiratory substrate, the medium was supplemented with 2 μM rotenone. AV-6-93 or diflurone did not affect TPP^+ binding to mitochondrial membranes or the electrode response.

2.8 Ca^{2+}-induced mitochondrial membrane transition pore (MPT)

Ca^{2+}-induced MPT was evaluated by measuring changes in mitochondrial transmembrane potential (Δψ) using a TPP^+ electrode, changes in oxygen consumption using a Clark-type electrode, and changes in Ca^{2+} fluxes using a Ca^{2+}-selective electrode. The reactions were conducted in a medium containing 200 mM sucrose, 10 mM Mops-Tris (pH 7.4), 1 mM KH_2PO_4, and 10 μM EGTA, supplemented with 2 μM rotenone, as previously described (Custódio et al., 1998a, 1998b). Mitochondria (1mg/ml) that were incubated at 30 °C for 3 min (in the absence and presence of AV-6-93 or diflurone) were energised with 10 mM succinate, and the single addition of Ca^{2+} (100 nmol/mg protein) was used to induce MPT. Control assays, in both the absence and presence of Ca^{2+} plus 0.75 nmol/mg protein cyclosporin A (CsA) and compound (when necessary) were also performed.

2.9 Lipid peroxidation

The extent of lipid peroxidation was evaluated by oxygen consumption using a Clark-type electrode at 30 °C in an open glass chamber equipped with magnetic stirring. Mitochondria (1 mg/ml) were pre-incubated for 3 min in a medium containing 175 mM KCl, 10 mM Tris–Cl (pH 7.4), supplemented with 3 μM rotenone (in the presence or absence of tested compounds) to avoid mitochondrial respiration induced by endogenous respiratory substrates. The iron solution was prepared immediately before use and was protected from light. The changes in O_2 tension were recorded in a potentiometric chart record and oxygen consumption was calculated assuming an oxygen concentration of 230 nmol/ml. Membrane lipid peroxidation was initiated by adding 1 mM ADP/0.1 mM Fe^{2+} as oxidizing agents. Controls, in the absence of ADP/Fe^{2+}, were performed under the same conditions.

Lipid peroxidation was also determined by measuring thiobarbituric acid reactive substances (TBARs), using the thiobarbituric acid assay (Ernster & Nordenbrand, 1967). Aliquots of mitochondrial suspensions (0.5 ml each), 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, then cooled on ice for 10 min, and centrifuged at 850 *g* for 10 min. Controls, in the absence of ADP/Fe^{2+}, were performed under the same conditions. The supernatant fractions were collected and lipid peroxidation was estimated

spectrophotometrically at 530 nm. As blanks, we used control reactions performed in the absence of mitochondria and ADP/Fe^{2+}. The amount of TBARs formed was calculated using a molar extinction coefficient of 1.56 x 10^5 mol^{-1} cm^{-1} and expressed as nmol TBARs/mg protein (Buege & Aust, 1978).

2.10 Statistical analysis

The cytotoxicity data were calculated as a mean ± S.E. Statistical analysis was performed using Student's t-test or one-way analysis of variance (ANOVA), followed by a Bonferroni multiple comparisons test.

The mitochondrial experiments were performed using three independent experiments with different mitochondrial preparations. The values are expressed as means ± S.E. Means were compared using one-way ANOVA for multiple comparisons, followed by Tukey's test. Statistical significance was set at $p< 0.05$.

3. Results

3.1 Protection against the cell death induced by MPP^+

In primary rat cortical neurones, AV-6-93 at concentrations of 1 and 10 μM decreased MPP^+-induced cell death by 75% and 56%, respectively (Fig. 2A). Diflurone exerted the protective ability only at the highest tested concentration, 10 μM, and decreased the MPP^+-induced cell death by 35% (Fig. 2B). Neither AV-6-93 nor diflurone, added without MPP^+, changed cell viability at the highest tested concentrations (Fig. 2).

3.2 Effects of AV-6-93 and diflurone on rat liver mitochondrial bioenergetics

AV-6-93 and diflurone (up to 100 μM) were studied for their effects on mitochondrial bioenergetics by evaluating several mitochondrial respiratory chain parameters (state 2, state 3, state 4, FCCP-stimulated respiration, RCR, ADP/O ratio, Δψ, and phosphorylation rate) using glutamate/malate as the respiratory substrate.

The effects of AV-6-93 on glutamate/malate-supported respiratory rates (state 2, state 3, state 4 and FCCP-stimulated respiration), respiratory indices RCR and ADP/O of rat liver mitochondria were almost non-existent and insignificant at concentrations of up to 100 μM (Table 1), indicating that the compounds did not significantly affect mitochondrial bioenergetics.

These results are demonstrated in Table 2, where AV-6-93 and diflurone, at concentrations of up to 100 μM, did not significantly affect either the Δψ induced by glutamate/malate-dependent respiration or the phosphorylation time.

As for glutamate/malate-supported respiration, the effects of AV-6-93 and diflurone on succinate-supported respiratory rates (state 2, state 3, state 4 and FCCP**)** and respiratory indices RCR and ADP/O of rat liver mitochondria were not significantly affected (results not shown), further supporting the finding that these compounds did not affect mitochondrial bioenergetics.

3.3 Effects of AV-6-93 and diflurone on Ca^{2+}-induced MPT

The effect of AV-6-93 and diflurone on Ca^{2+}-induced MPT was studied in order to evaluate their capacity to protect mitochondria against MPT opening by measuring the decrease in Δψ, the increase in oxygen consumption, and the Ca^{2+}-induced release of mitochondrial Ca^{2+}, which are typical phenomena that follow the induction of MPT. The amount of Ca^{2+} used to induce MPT was 100 nmol/mg protein.

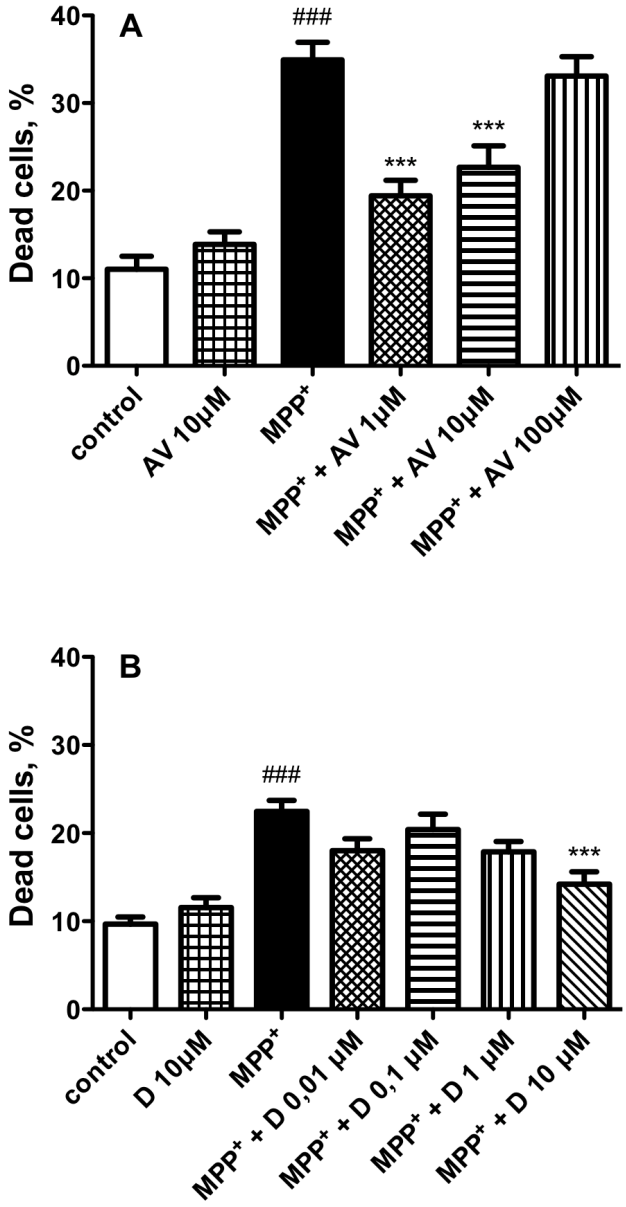

Fig. 2. Influence of AV-6-93 (AV) and diflurone (D) on MPP^+-induced cell death in primary rat cortical neurons (A and B, respectively). Cell death measured by Trypan blue method. Data are presented as a mean ± S.E. ### $p < 0.001$ vs control, t-test, *** $p < 0.001$ vs MPP^+, one-way ANOVA followed by Bonferroni multiple comparison's test.

Compounds (μM)	Oxygen consumption (% of control)				RCR	ADP/O
	State 2	State 3	State 4	State FCCP		
AV-6-93						
0.0	100.0 ± 0.0	100 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	6.8 ± 1.3	3.01 ± 0.1
1.0	100.0 ± 0.0	113.4 ± 6.7	94.4 ± 5.6	100.2 ± 8.7	6.5 ± 1.5	2.9 ± 0.1
10.0	105.0 ± 18.9	104.0 ±7.3	119.4 ± 10.0	101.9 ± 7.3	6.4 ± 1.0	2.8 ± 0.4
100.0	127.2 ± 31.9	109.9 ± 6.6	145.8 ± 44.5	91.2 ± 8.5	6.3 ± 2.7	2.8 ± 0.1
Diflurone						
0	100.0± 0.0	100.0± 0.0	100.0± 0.0	100.0± 0.0	6.8 ± 1.3	3.01 ± 0.1
100	107.3 ± 4.8	95.9 ± 2.9	104.4 ± 5.27	94.4 ± 4.03	6.4 ± 1.2	2.9 ± 0.09

Table 1. Effects of AV-6-93 and diflurone on the respiratory parameters (state 2, state 3, state 4, FCCP-stimulated respiration) and respiratory indices (RCR and ADP/O ratio) of rat liver mitochondria using glutamate/malate as respiratory substrate.

The values, which are given in percentage of control (% of control), correspond to the mean ± S.E. of the respiratory parameters, evaluated in three different mitochondrial preparations, at the different indicated situations. Control values are expressed in nmol O_2**.** mg^{-1} protein min^{-1}: state 2 = 7.1 ± 0.7; state 3 = 39.4 ± 4.0; state 4 = 5.62 ± 0.8; FCCP-stimulated respiration = 57.14 ± 10.4.

Compounds (μM)	Δψ (mV)			Phosphorylation time (s)
	Glu/Mal energisation	ADP depolarisation	Repolarisation	
AV-6-93				
0	-220.5 ± 5.1	21.7 ± 2.2	-216.8 ± 2.6	33.0 ± 3.0
1	-219.0 ± 4.0	21.3 ± 1.9	-217.0 ± 4.3	32.7 ± 2.9
10	-220.6 ± 4.1	23.5 ± 2.1	-218.7 ± 4.2	32.5 ± 4.5
100	-216.0 ± 5.6	23.4 ± 1.1	-212.5 ± 4.7	39.0 ± 1.7
Diflurone				
0	-220.5 ± 5.1	21.7 ± 2.2	-216.8 ± 2.6	33.0 ± 3.0
100	-218.0 ± 1.7	21.0± 0.3	-216.0 ± 1.4	32.8 ± 2.3

Table 2. Effects of AV-6-93 and diflurone on glutamate/malate-dependent transmembrane potential (Δψ) and phosphorylation time of rat liver mitochondria.

The values correspond to the mean ± S.E. of the Δψ and the phosphorylation time, evaluated in three different mitochondrial preparations, at the different indicated situations.
The results of the effect of AV-6-93 on MTP protection are depicted in Fig. 3. Under control conditions, the addition of 10 mM succinate to mitochondrial suspensions produced a Δψ of about -216 mV (negative inside mitochondria) (Fig. 3A), corresponding to respiratory state 4 (Fig. 3B). The addition of Ca^{2+} led to a rapid depolarisation (decrease of Δψ), followed by a partial repolarisation (recover of Δψ), the subsequent total depolarisation of mitochondria (Fig. 3A), and an increase in respiratory state 4 (Fig. 3B).

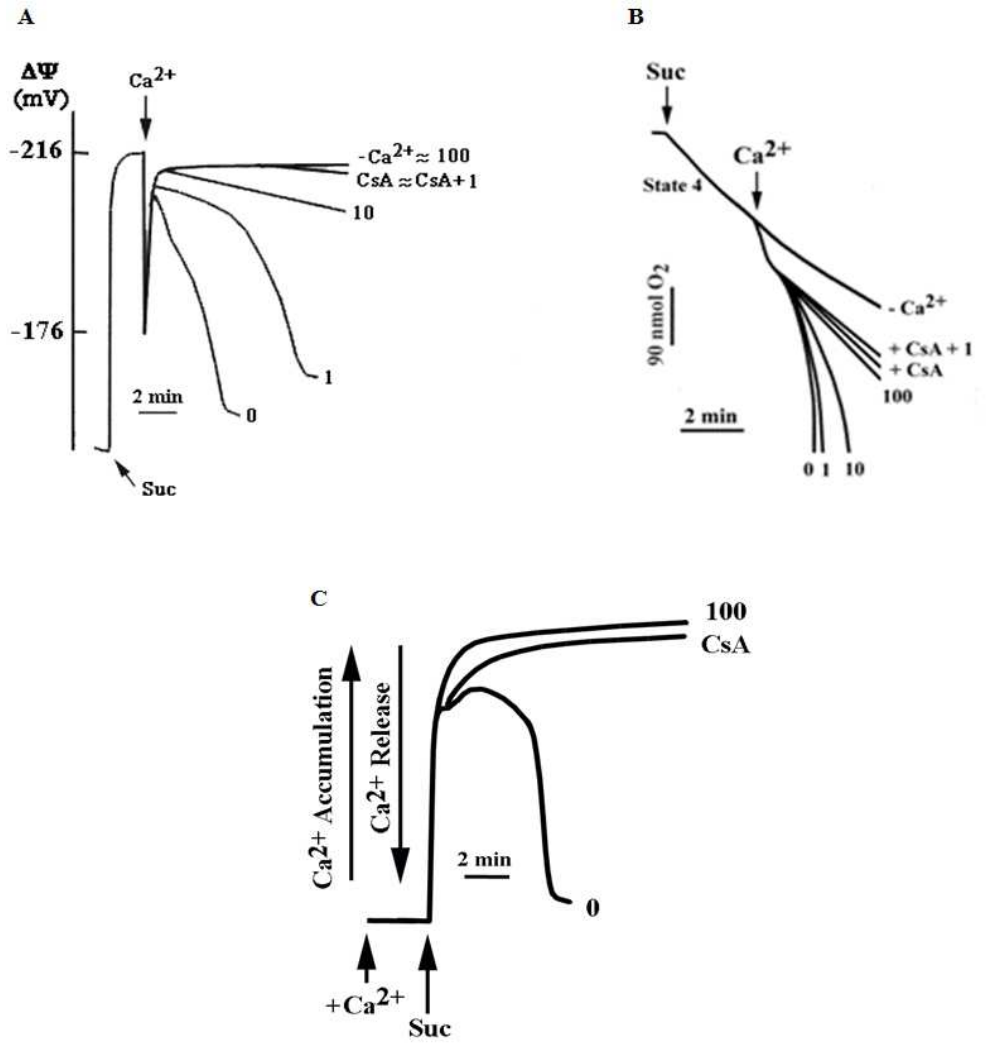

Fig. 3. Effect of AV-6-93 on rat liver MPT induced by Ca^{2+}.

Evaluation was performed by measuring succinate-supported transmembrane potential ($\Delta\psi$) (A), oxygen consumption (B), and mitochondrial Ca^{2+} fluxes (C). Additions of 100 nmol calcium/mg protein (Ca^{2+}) and 10 mM succinate (Suc); additions of AV-6-93 at the concentrations of 1, 10, and 100 μM (1, 10, 100) are indicated. Assays in the absence of Ca^{2+} (-Ca^{2+}); assays in the presence of Ca^{2+} plus CsA (0.75 nmol/mg protein (CsA); assays in the presence of Ca^{2+} plus CsA + 1μM AV-6-93 (CsA+ 1). The traces are representative of assays with three different mitochondrial preparations.

These effects were due to the entry of Ca^{2+} into the electronegative mitochondrial matrix (Fig. 3C), followed by the efflux of H^+ for restoring the $\Delta\psi$. Incubation of mitochondria with

AV-6-93 concentrations of up to 100 μM for 3 min before energisation with succinate prevented total depolarisation of mitochondria (Fig. 3A), the increase in respiratory state 4 (Fig. 3B), and the release of mitochondrial Ca^{2+} (Fig. 3C), suggesting that this compound has a high ability to protect mitochondria against MPT induction. Incubation of mitochondria with 0.75 nmol/mg protein, CsA, a specific inhibitor of MPT (Broekemeier et al., 1989), for 2 min before energising with succinate, either in the absence or presence of 1 μM AV-6-93, completely blocked mitochondrial depolarisation (Fig. 3A), the increase in respiratory state 4 (Fig. 3B), and the Ca^{2+}-induced release of mitochondrial Ca^{2+} (Fig. 3C). These data show that these effects had been induced by MPT. In contrast to AV-6-93, diflurone, in the same concentration range, did not prevent either the depolarisation of mitochondria or the release of mitochondrial Ca^{2+} (results not shown), indicating that this compound did not protect mitochondria against MPT.

3.4 Effects of AV-6-93 and diflurone on mitochondrial oxidative stress

The effects of AV-6-93 and diflurone on mitochondrial oxidative damage were assessed by detecting the mitochondrial membrane lipid peroxidation induced by the pro-oxidant pair ADP/Fe^{2+}. Lipid peroxidation was evaluated by measuring oxygen consumption (Fig. 4) and TBARs formation (Table 3). In the absence of AV-6-93 and after the addition of the pro-oxidant pair, it is possible to distinguish two-phase kinetics in oxygen consumption: an initial lag phase, characterized by slow oxygen consumption lasting about 2 min, is followed by a rapid oxygen consumption phase. The lag phase is probably related with 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 reactive oxygen species (ROS) and, consequently, due to the propagation phase of lipid peroxidation (Sassa et al., 1990). AV-6-93 concentrations up to 100 μM enlarged the lag phase of slow oxygen consumption before the oxygen uptake burst induced by the ADP/Fe^{2+} complex and increased the rate of the rapid oxygen consumption phase (Fig. 4), suggesting that the compounds affected both the initiation and the propagation of lipid peroxidation of mitochondrial membranes.

These results agree with the quantitative evaluation of TBARs formation performed to confirm the protective effects of AV-6-93. The data in Table 3 show that the kinetics of TBARs formation induced by ADP/Fe^{2+} are similar to that observed for oxygen consumption. The same range of AV-6-93 concentrations used in the oxygen consumption assays also affected TBARs formation. TBARs formation in the absence of ADP/Fe^{2+} was negligible (0.44 ± 0.25 nmol/mg of protein). In contrast to AV-6-93, diflurone, in the same concentration range, did not affect oxygen consumption induced by the ADP/Fe^{2+} complex or TBARs formation (results not shown), indicating that this compound has no capacity to protect mitochondria against the lipid peroxidation induced by the pro-oxidant pair ADP/Fe^{2+}.

Lipid peroxidation was evaluated by oxygen consumption and initiated by adding 1 mM ADP/0.1 mM Fe^{2+} to mitochondrial suspensions (Fig. 4). The traces represent typical direct oxygen consumption recordings of three experiments obtained from different mitochondrial preparations; controls in the absence of ADP/Fe^{2+} (–ADP/Fe^{2+}); assays in the presence of AV-6-93 at the concentrations 1, 10, 20, 50, 100 μM (1, 10, 20, 50, 100).

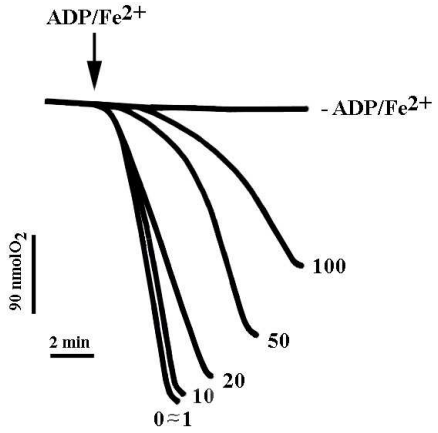

Fig. 4. Effect of AV-6-93 on membrane lipid peroxidation of rat liver mitochondria induced by the pro-oxidant pair ADP/Fe^{2+} evaluated by oxygen consumption.

AV-6-93 (μM)	TBARs (nmol/mg protein/10 min)
0	10.2 ± 1.0
1	9.6 ± 1.0
10	8.5 ± 0.5
20	7.6 ± 1.0
50	5.2 ± 2.1
100	3.1± 1.9*

Table 3. Effect of AV-6-93 on membrane lipid peroxidation of rat liver mitochondria induced by the pro-oxidant pair ADP/Fe^{2+} evaluated by TBARs assay. The data correspond to the mean ± S.E. of three independent experiments.
*$p < 0.05$ vs control (in the absence of AV-6-93).

4. Discussion

Studies examining the importance of mitochondrial pathophysiology in neurodegeneration provide a target for additional treatments with agents that improve mitochondrial function, protect MPT, and/or exert antioxidant activity (Petrozzi et al., 2007). These studies lead to novel approaches in the treatment of neurodegenerative diseases, such as Parkinson's disease, with disease-modifying drugs.

The aim of the present study was to examine the abilities of two novel adamantane-containing DHP analogues, AV-6-93 and diflurone, to protect against cell death induced by mitochondrial toxin MPP^+ and beneficially influence mitochondrial processes in an attempt to identify putative antiparkinsonian drugs.

First, we examined how both compounds acted in primary cortical cultures in response to MPP^+. AV-6-93, at concentrations of 1 and 10 μM, significantly protected against MPP^+-induced cell death by 75% and 56%, respectively, whereas diflurone protected against cell death by 35% at a concentration of 10 μM. Neither AV-6-93 nor diflurone, added without MPP^+, changed cell viability.

A larger difference between the compounds' activities was observed in isolated rat liver mitochondria by the assessment of their ability to affect both the Ca^{2+}-induced mitochondrial permeability transition (MPT) and lipid peroxidation. To assess the Ca^{2+}-induced MPT, the evaluation of the drop of $\Delta\psi$, the increase in mitochondrial respiration associated with Ca^{2+} accumulation in the mitochondrial matrix, and the mitochondrial Ca^{2+} fluxes were carried out. Changes in these parameters help us to conclude whether the compound protects mitochondria against MPT induction and, consequently, to discern whether the compound alters mitochondrial Ca^{2+} homeostasis. AV-6-93, at a concentration of 10 μM, significantly protected mitochondria against MTP induction and provided complete protection at 100 μM, as revealed by its ability to prevent the depolarisation of mitochondria, the increase in mitochondrial respiration and mitochondrial Ca^{2+} release. These effects were comparable with that of CsA (0.75 nmol/mg protein), a specific inhibitor of the mitochondrial permeability transition pore. Diflurone was ineffective in these tests. The effectiveness of AV-6-93 can be considered to be very promising because it indicates the ability of this compound to halt mitochondrial swelling and cell death, both consequences of the induction of the permeability transition pore.

A critical factor for induction of MPT is the oxidation of thiol groups of the MPT complex, creating diethyl cross-links (Costantini et al., 1996; 1998, Halestrap et al., 1997; McStay et al., 2002). Therefore, the most plausible hypothesis to explain the partial MPT protection induced by AV-6-93 is that changes in the redox-state of thiol groups of the MPT complex is provided via avoiding of diethyl cross-links. This hypothesis is supported by the observation that AV-6-93 protected mitochondria against oxidative stress. Oxidative stress was assessed by evaluating the extent of lipid peroxidation by measuring oxygen consumption and TBARs formation. Alterations of these parameters may reveal whether the compound protects mitochondria against oxidative stress, i.e., whether the compound acts as an antioxidant. AV-6-93, at concentrations up to 100 μM, protected (by about a half) mitochondria against membrane lipid peroxidation, as inferred by its ability to inhibit both oxygen consumption and TBARs formation induced by the pro-oxidant pair ADP/Fe^{2+}. These data suggest that this compound may act as antioxidant because it can avoid both the initiation and the propagation of the oxidation of polyunsaturated fatty acid acyl chains of membrane phospholipids induced by the perferryl ion complex ADP-Fe^{3+}-O_2^-, a mechanism suggested to be responsible for lipid peroxidation (Sassa et al., 1990). In contrast to AV-6-93, diflurone, under the same conditions, had no capacity to protect mitochondria against oxidative damage induced by the pro-oxidant pair ADP/Fe^{2+}.

The only common feature of both compounds was a lack of influence on mitochondrial bioenergetics, which was assessed by analysing several mitochondrial functioning parameters of the respiratory chain (respiration states 2, 3, 4, FCCP-stimulated respiration, the RCR, and the ADP/O ratio) and the oxidative phosphorylation system ($\Delta\psi$ and phosphorylation time), using both glutamate/malate and succinate as respiratory substrates. According to the mitochondrial parameters affected, it is possible to assess how the compound interferes with mitochondrial bioenergetics: by perturbing the permeability (integrity) of the inner mitochondrial membrane (stimulation of respiration states 2 and 4), by impairing the respiratory chain (inhibition of FCCP-stimulated respiration), and/or by acting at the level of the phosphorylation system (affecting respiration state 3). Both AV-6-93 and diflurone, at concentrations of up to 100 μM, failed to significantly affect liver mitochondrial bioenergetics, as shown by the lack of effects on both glutamate/malate- and succinate-supported respiration in state 2, state 3, state 4, FCCP-stimulated respiration, RCR and ADP/O ratios, $\Delta\psi$ and phosphorylation time.

To address why both adamantane-containing compounds showed very distinct effects on mitochondrial damage induced by both Ca^{2+} and ADP/Fe^{2+}, one may suggest that the molecular "volume" of AV-6-93 (one adamantane ring-containing DHP) is more optimal than that of diflurone (two adamantane ring-containing DHP) for mitochondrial protection. The two adamantane rings in the diflurone molecule probably generate a steric hindrance that prevents or delays the chemical reaction, which can easily occur in the case of AV-6-93, a one adamantine ring-containing DHP.
Based on the results obtained in primary cortical cultures, the two-adamantane DHP structure is not as crucial as it is in isolated rat liver mitochondria because diflurone has not lost its activity to prevent cell death caused by MPP^+ (a toxin focused on mitochondrial complex I). However, the activity of diflurone was lower than that of AV-6-93. One could suggest that, in addition to the protection of complex I, other cellular signalling mechanisms may be initiated by DHP compounds to increase cell survival.

5. Conclusion

The novel one-adamantane 1,4-dihydropyridine compound AV-6-93 is capable of regulating cell survival processes with regards to mitochondrial processes, such as inhibition of the induction of the permeability transition pore and prevention of oxidative stress. The effectiveness of AV-6-93 can be considered to be very promising in the treatment of neurodegenerative diseases associated with compromised mitochondrial processes, e.g., Parkinson's disease.

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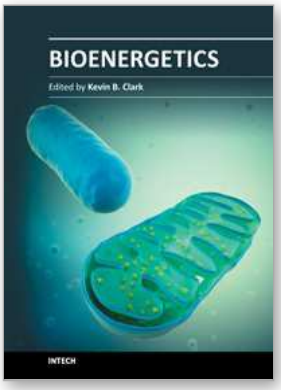

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Cellular life depends upon energy storage, transformation, utilization, and exchange in order to optimally function and to stay-off death. The over 200-year-old study of how cells transform biological fuels into usable energy, a process broadly known as bioenergetics, has produced celebrated traditions in explaining origins of life, metabolism, ecological adaptation, homeostasis, biosynthesis, aging, disease, and numerous other life processes. InTech's edited volume, Bioenergetics, brings together some of these traditions for readers through a collection of chapters written by international authorities. Novice and expert will find this book bridges scientific revolutions in organismic biology, membrane physiology, and molecular biology to advance the discipline of bioenergetics toward solving contemporary and future problems in metabolic diseases, life transitions and longevity, and performance optimization.

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