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Mitochondrial respiratory chain as a new target for anti-ischemic molecules

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Abstract

Vascular diseases like thrombosis, myocardial infarction, cerebral ischemia or chronic venous insufficiency affect a high proportion of the population. They are all associated with more or less pronounced ischemic conditions. We have previously shown that some venotropic drugs display an anti-ischemic activity, i.e. they prevent the hypoxia-induced decrease in ATP content in cultured cells. The effect is due to the fact that these molecules maintain mitochondrial respiratory activity during hypoxia. Among them is bilobalide. Starting from the 3D structure of bilobalide, we designed new molecules presenting the same chemical features. They were synthesized and tested for their biological activity. As the parent compound, two of them, malonic acid dicyclopent-2-enyl ester (MRC2P119) and 2-oxo-3-oxa-bicyclo[3.1.0]hexane-1-carboxylic acid allyl ester (MRC2P57), were able to markedly increase the respiratory control ratio of isolated mitochondria. They are able to prevent the inhibition of complex I by amytal and of complex III by myxothiazol, but not the uncoupling of the respiration by carbonylcyanide *m*-chlorophenyl hydrazone (*m*-CCP). Moreover, MRC2P119 and MRCP2P57 inhibit, in a dose-dependent way, the hypoxia-induced decrease in ATP content in endothelial cells as well as the subsequent activation of these cells as evidenced by an inhibition of the increase in neutrophil adherence to the endothelial cells induced by hypoxia. Finally, MRC2P119 prevent the hypoxia- and the hypoxia–reoxygenation-induced decrease in viability of SH-SY5Y neuroblastoma cells. In conclusion, we identified two new molecules, which display anti-ischemic properties when tested in vitro on endothelial and neuronal cell types. This anti-ischemic activity is probably due to a protection of complexes I and III of the mitochondrial respiratory chain.

Keywords: Anti-ischemic molecule; Hypoxia; Mitochondrion; Respiration; Complex I; Endothelial cell

1. Introduction

Vascular diseases affect a high proportion of the population in developed countries. They vary in nature including such conditions as thrombosis, atherosclerosis, myocardial infarction, cerebral ischemia and chronic venous insufficiency. All of these diseases are associated with more or less pronounced ischemic conditions.

Mitochondria are the main source of energy, which sustains cellular metabolism and integrity. The decrease in oxygen supply during ischemia impairs energy production by these organelles. Since long, it has been established that, in a large number of organs, there is a relationship between mitochondrial dysfunction and the irreversibility of pathological damage (Veitch et al., 1992; Allen et al., 1995). Indeed, the transition from reversible to irreversible ischemia is probably dependent on the functional state of mitochondria (Jennings, 1969; Taegtmeyer et al., 1985).

Mitochondria dysfunction in ischemic conditions can be attributed mainly to either inhibition of adenine nucleotide translocase (Duan and Karmazyn, 1989), to a reduced activity of complex I and III (Veitch et al., 1992; Allen et al., 1995), to alteration of the membrane fluidity (Sun and Gilboe, 1994) or to calcium overload (Silverman, 1993). We previously showed that some venotropic drugs (aescine, diosmin, naphthoquinone, Ginkor Fort) as well as naftidrofuryl protect complexes I and III of the respiratory chain, while others (hydroxyethylrutosides, procyanidolic oligomers) are able to increase adenine nucleotide translocase activity, hence increasing the mitochondrial respiratory activity (Janssens et al., 2000a). These results, thus, suggest that at least part of the therapeutical benefit of these drugs would be to protect mitochondria in ischemic conditions,

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thus preventing the decrease in cellular ATP content induced by hypoxia and hence delaying the pathological activation cascade which then occurs in these cells. Indeed, such a protective effect of the hypoxia-incubated endothelial cells has been well demonstrated for Ginkor Fort (Arnould et al., 1998), hydroxyethylrutosides (Janssens et al., 1996), aescine (Arnould et al., 1996) and naftidrofuryl (Michiels et al., 1993). Protection or delay of cell death in ischemic conditions would also be predicted if the decrease in ATP content is delayed.

Many of these drugs are derived from plant extracts and the active compounds of some of these extracts are not identified. The aim of this work was to synthesize new molecules which would have the same chemical features than these compounds as well as the same biochemical effects in order to create a new generation of anti-ischemic molecules easily available and possibly more potent since designed for a specific target, i.e. the mitochondrial respiratory chain.

2. Materials and methods

2.1. Isolation of rat liver mitochondria

Female Wistar rats (IFFA Credo, Brussels, Belgium) were housed before the experiments in groups of four and allowed to acclimatize to their new laboratory conditions for at least 14 days. Rats were fasted for at least 18 h and after sacrification, the liver was chilled in a medium containing 0.25 M saccharose, 1 mM EDTA, 1 mM HEPES and 2 g/l bovine serum albumin. 3 g of liver were homogenized by two successive passages in a Teflon homogenize (Type C, AH Thomas, Philadelphia, PA). A nuclear fraction was prepared by a 10 min centrifugation at $754 \times g$ at 4 °C. The supernatant was kept at 4 °C. The pellet was centrifuged again 10 min at $580 \times g$ and the supernatant added to the previous one and adjusted to a final volume of 45 ml. Two times 8 ml were sampled to isolate mitochondria by a 3-min centrifugation at $10300 \times g$ (Beckman LS65B Ultracentrifuge, Beckman Instrument, Spinco Div., Palo Alto, CA). Resuspension of the mitochondrial pellet was carried out carefully with a 7-ml Dounce loose glass homogenizer (Kontes Glass, Vineland, NY) in NaCl 7.05 mM, KCl 70.5 mM, K_2HPO_4 5.45 mM, KH_2PO_4 4.55 mM, bovine serum albumin 0.15%, pH 7.2.

2.2. Mitochondrial incubation with the drugs

Mitochondria isolated from rat liver were suspended in NaCl 7.05 mM, KCl 70.5 mM, K_2HPO_4 5.45 mM, KH_2PO_4 4.55 mM, bovine serum albumin 0.15%, pH 7.2. at a final mitochondrial protein concentration of approximately 40 mg/ml. The mitochondria were then incubated for 60 min in the presence of the different drugs at 4 °C. At the end of the incubation, state 3 and state 4 of the respiration were measured.

2.3. Respiration determination

The rate of oxygen consumption by the mitochondrial fraction was assayed by an oxypolarographic method using a Clark-type electrode. The respiratory control ratio (RCR) was calculated as follows: it is the ratio between the oxygen consumption rate in the presence of exogenous succinate 5 mM and ADP 0.16 mM (state 3) and the rate after ADP consumption (state 4). The values for state 2 and state 4 respiratory rates were similar in our experimental conditions.

2.4. Inhibition of the mitochondrial electron transfer

Oxygen consumption was followed by a Clark electrode. Reactions were performed at 25 °C in a 2.5 ml chamber containing 2 mg of mitochondrial suspension in the incubation buffer. Respiration rates were measured using 10 mM D-β-hydroxybutyrate (for complex I inhibition) or 5 mM of succinate (for complex III inhibition or for uncoupling with carbonylcyanide *m*-chlorophenyl hydrazone, *m*-CCP) as substrate. Inhibition studies were performed by the addition of amytal at 0.36 mM (for complex I), of myxothiazol at 0.288 μM (for complex III) or of *m*-CCP at 0.5 μM (for uncoupling) to a reaction containing unlimiting amounts of substrate.

The experiment was performed as follow: O_2 consumption is measured for mitochondria in the presence of the substrate, then the inhibitor is added into the chamber and O_2 consumption is again measured. The inhibition is, thus, measured on state 4 respiration rate. This is done both for control mitochondria and for mitochondria preincubated with the molecule. The percentage of inhibition is calculated as "$100 \times$ (O_2 consumption without inhibitor $-O_2$ consumption with inhibitor)/O_2 consumption without inhibitor".

2.5. Measurement of complex I activity

The activity of NADH cytochrome *c* reductase was measured according to Boffoli et al. (1994). Mitochondria were frozen at −70 °C, thawed, 23 μl of the suspension were diluted in 1 ml of 25 mM potassium phosphate buffer, 5 mM $MgCl_2$, pH 7.4 containing 10 μM cytochrome *c* and 2.5 mg/ml bovine serum albumin and sonicated 30 s at 4 °C. Then 2 mM KCN was added, the mitochondria were incubated 5 min at 37 °C and the reaction was initiated by the addition of 2.5 mM NADH. The reduction of cytochrome *c* was followed at 550 nm at 37 °C. In order to subtract the reduction of cytochrome *c* which was independent of the complex I activity, the same reaction was measured in the presence of rotenone 2 μg/ml.

2.6. Endothelial cell isolation and culture

Human umbilical vein endothelial cells were isolated according to Jaffe et al. (1973). Cords were stored at 4 °C just after birth in stock buffer (4 mM KCl, 140 mM NaCl,

10 mM HEPES, 1 mM glucose, 100 μg/ml streptomycin, 100 U/ml penicillin and 0.25 μg/ml fungizone). Before manipulation, they were rinsed with 20 ml phosphate-buffer saline (PBS) containing antibiotics and fungizone at concentrations aforementioned. Umbilical veins were incubated for 35 min at 37 °C with 4 ml collagenase type II (Sigma, St. Louis, MO, USA) 0.05% in PBS. The cells were then harvested in M199 (Gibco, Paisley, Scotland) + 20% fetal calf serum (Gibco), centrifuged 10 min at 1000 rpm, and seeded in 0.20% gelatin-coated culture dishes (25 cm^2, Falcon Plastics, Oxnard, CA). The following day, the cells were washed with medium to eliminate blood cell contamination. Only primary cultures were used for these studies. Confirmation of their identity as endothelial cells was obtained by detecting factor VIII antigen assessed by immunofluorescence staining.

2.7. SH-5YSY culture

The human neuroblastoma cells line SH-5YSY was cultivated in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum.

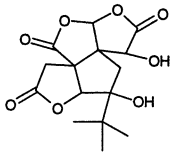
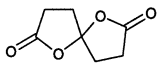
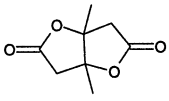
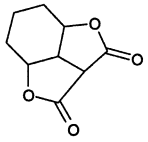
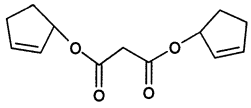
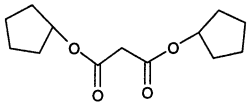
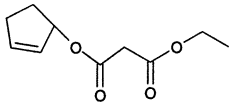
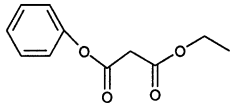
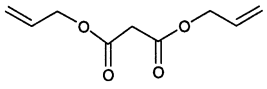
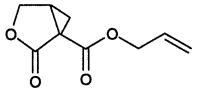
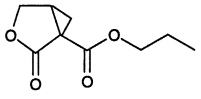
2.8. In vitro model of hypoxia

Hypoxia incubation was performed by exposing cells to 100% N_2 at 37 °C. PO_2 was 130 mm Hg under normal conditions, decreasing to 10 mm Hg after 30 min hypoxia (Michiels et al., 1992). Human umbilical vein endothelial cells were seeded in gelatine-coated Petri dishes (35 mm, Falcon Plastics). For incubation, cells were rinsed twice with Hank's balanced salt solution (HBSS) and covered with 0.7 ml of HBSS. Medium was reduced to a uniform thin layer to decrease the diffusion distances of atmospheric gases. Modified HBSS (140 mM NaCl, 5 mM KCl, 0.4 mM $MgSO_4 \cdot 7H_2O$, 0.5 mM $MgCl_2 \cdot 6H_2O$, 3 mM $Na_2HPO_4 \cdot 2H_2O$, 0.4 mM KH_2PO_4, 5.5 mM glucose, pH 7.35) was prepared in our laboratory. Two hours of hypoxia was chosen because it is the maximal time that endothelial cells can sustain without loss of viability. SH-5YSY were seeded at 50,000 cells/well (24-well plate, Corning). Cells were covered with 1 ml CO_2-independent medium (Gibco) containing 2% fetal calf serum and incubated 24 h in hypoxia. Afterwards, medium was replaced by 1 ml DMEM containing 10% fetal calf serum for 24-h reoxygenation in normoxic conditions. MRC2P119 was present during both hypoxia and reoxygenation. Corresponding controls were always performed with cells incubated under the same conditions but kept in normoxia (ambient atmosphere).

2.9. ATP assay

ATP assay was performed using a bioluminescent ATP assay kit (FL-ASC, Sigma) using luciferase and luciferine. After incubation under normoxia or hypoxia, cells were lysed with 0.5 ml of somatic cell ATP-releasing reagent (Sigma) for a few seconds, and the supernatant recovered for the assay performed in a luminometer (Biocounter M2010, Lumac, Landgraaf, The Netherlands). The absolute

Table 1
Structure and effect of bilobalide and its related molecules on mitochondrial respiration

	bilobalide	33% at $2.5\ 10^{-6}$M
	1,5-dioxospiro-2,6-nonanedione [4:0:4]	no effect
	2,6-dimethyl-hexa-hydrofuro-furan-4,8-dione	no effect
	BN50504	12% at 10^{-6}M
	MRC2P119	21% at 10^{-6}M
	MRC2P141	no effect
	MRC2P45	10% at 10^{-5}M
	MRC3P133	no effect
	MRC2P55	no effect
	MRC2P57	26% at 10^{-7}M
	MRC3P47	8% at 10^{-6}M

Liver mitochondria were purified from nontreated rats. Mitochondria were then incubated for 1 h with different concentrations of the molecules or with mitochondria incubation medium (control) and Respiratory Control Ratio (RCR) was measured in the presence of succinate. Results are expressed in percentage of increase in RCR compared to control.

values of ATP yielded from the experiments were expressed in relative light unit (RLU)/μg of proteins. The amount of proteins was assayed for each test to correct the amount of ATP measured in the bioluminescent assay with the number of cells present in each dish.

2.10. Neutrophil adherence to endothelial cells

Human neutrophils were purified from blood of healthy donors as previously described (Arnould et al., 1993). In brief, 30 ml of venous anticoagulated blood from normal subjects was mixed with 5 ml of 6% dextran (Pharmacia Fine Chemicals, Uppsala, Sweden) and allowed to sediment at 20 °C for 60 min. After hypotonic lysis of erythrocytes performed with NaCl 0.2% for 1 min, the cells were centrifuged 20 min at 1000 rpm on Lymphoprep (Nycomed Pharma, Oslo, Norway). For labeling, neutrophils at the density of 10×10^6 cells/ml were incubated with 20 μCi ^{51}Cr/ml (specific activity = 250–500 mCi/mg chromium, Amersham Laboratories, Buckinghamshire, UK) in HBSS with calcium and magnesium for 60 min at room temperature, washed three times and then suspended at 5×10^6 cells/ml in HBSS.

Endothelial cells were seeded at confluence in Petri dishes. After hypoxia incubation, HBSS was removed and 1 ml of ^{51}Cr-labeled neutrophils was added on the endothelial cell monolayer. After a 5-min coincubation at 37 °C, dishes were washed three times with 0.5 ml HBSS to remove nonadherent neutrophils. The remaining adherent neutrophils were then solubilized with 0.5 ml NaOH 1 N and the radioactivity was measured in a gamma counter.

2.11. Lactate dehydrogenase release

After the incubation, the medium was recovered and lactate dehydrogenase (LDH) activity was assayed with the Cytotoxicity Detection Kit (Roche, Mannheim, Germany).

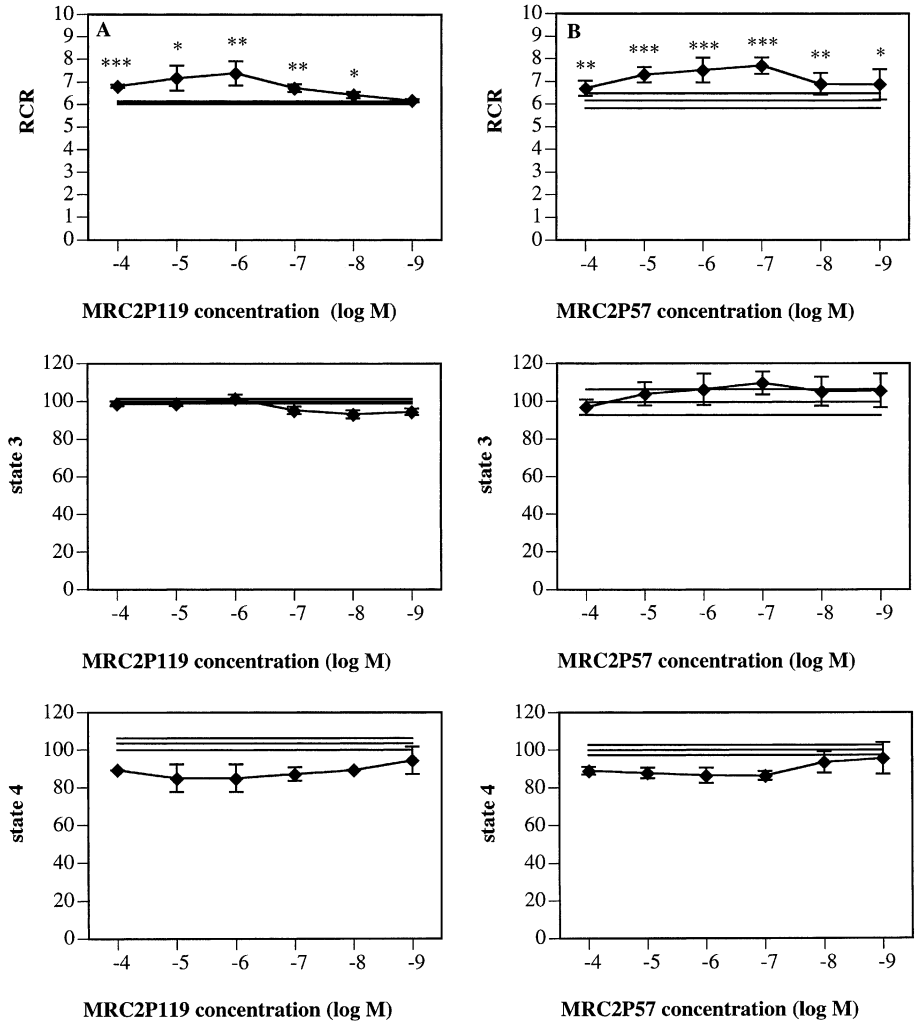


Fig. 1. Effect of MRC2P119 (A) or MRC2P57 (B) on mitochondrial respiration. Liver mitochondria were purified from nontreated rats. Mitochondria were then incubated for 1 h with different concentrations of the molecules or with mitochondria incubation medium (control), state 3 and state 4 respiration rates were measured in the presence of succinate and Respiratory Control Ratio (RCR) calculated. Results are expressed as means ± S.D. ($n = 3$ for A and $n = 6$ for B). The three horizontal lines represent the mean ± S.D. for control mitochondria. *, ** or ***: $P < 0.05$, $P < 0.01$ or $P < 0.001$ from control.

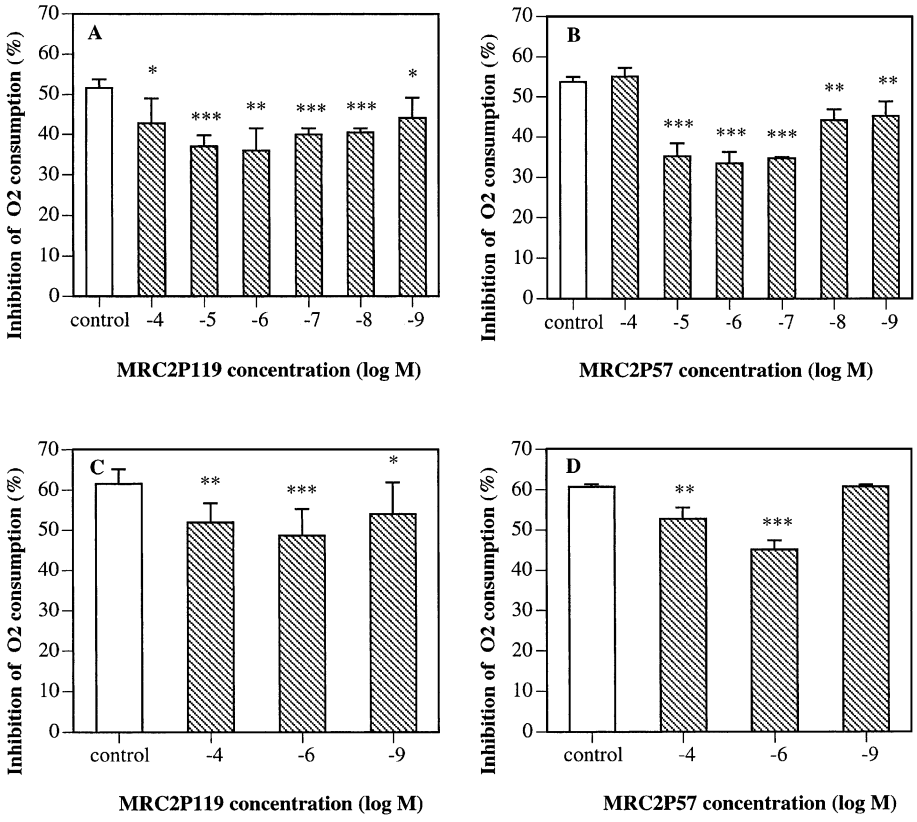


Fig. 2. Effect of MRC2P119 (A, C) or MRC2P57 (B, D) on the inhibition of complexes I and III. Liver mitochondria were purified from nontreated rats. Mitochondria were then incubated for 1 h with different concentrations of the molecules or with mitochondria incubation medium (control). O_2 consumption (CO) was then measured in the presence of amytal 0.36 mM (A, B) or in the presence of myxothiazol 0.288 μM (C, D). Results are expressed in percentage of inhibition of oxygen consumption as means ± S.D. ($n=3$ for A, B and d, $n=6$ for C). *, ** or ***: $P<0.05$, $P<0.01$ or $P<0.001$ from control.

2.12. Drugs

ADP, β-hydroxybutyrate, succinate, amytal, ferrocytochrome *c*, *m*-CCP, myxothioazol, rotenone, NADH and bovine serum albumin (A7030) were from Sigma. KCN and the other chemicals of analytical grade were from Merck (Darmstadt, Germany). Dioxyaspirononanedione came from Aldrich, dimethyl-hexahydrofuro-furan-dione was kindly provided by Prof. J. Kagan, University of Illinois, Chicago and hexahydro-2a,4a,5,6,7,7a 2*H*-furo [4,3,2-cd] benzofurane dione-2,3 (BN50504) by Institut Henri Beaufour, Les Ullis, France.

Malonic acid cyclopent-2-enyl ester ethyl ester (MRC2 P45), malonic acid diallyl ester (MRC2P55), 2-oxo-3-oxabicyclo[3.1.0]hexane-1-carboxylic acid allyl ester (MRC2 P57), malonic acid dicyclopent-2-enyl ester (MRC2P119), malonic acid dicyclopentyl ester (MRC2P141), 2-oxo-3-oxabicyclo[3.1.0]hexane-1-carboxylic acid propyl ester (MRC3

Table 2
Effect of MRC2P119 and MRC2P57 on the inhibition of oxygen consumption by uncoupling

	MRC2P119	MRC2P57
Control	61.9 ± 8.4	50.0 ± 6.8
10^{-3} M	64.3 ± 9.6	51.4 ± 13.5
10^{-4} M	57.9 ± 9.0	51.0 ± 10.5
10^{-5} M	66.9 ± 15.9	48.3 ± 4.5
10^{-6} M	69.0 ± 8.2	54.1 ± 6.2

Liver mitochondria were purified from nontreated rats. Mitochondria were then incubated for 1 h with different concentrations of MRC2P119 or MRC2P57 or with mitochondrial incubation medium (control). O_2 consumption was then measured in the presence of 0.5 μM m-CCP. Results are expressed in percentage of inhibition of oxygen consumption as means ± S.D. ($n=4$, except for control $n=5$).

Table 3
Effect of MRC2P119 and MRC2P57 on complex I activity

	MRC2P119	MRC2P57
Control	100 ± 5	100 ± 16.7
10^{-3} M	105 ± 10	90.6 ± 23.2
10^{-4} M	125 ± 15[a]	100.7 ± 10.9
10^{-5} M	140 ± 15[b]	115.2 ± 9.4
10^{-6} M	130 ± 5[c]	213.0 ± 65.2[a]
10^{-7} M	ND	95.6 ± 10.1

Liver mitochondria were purified from nontreated rats. Mitochondria were then incubated for 1 h with different concentrations of MRC2P119 or MRC2P57 or with mitochondrial incubation medium (control). Complex I activity was then measured. Results are calculated in U/mg proteins and expressed in percentage of control as means ± S.D. ($n=3$).
ND: not determined.
[a] $P<0.05$.
[b] $P<0.01$.
[c] $P<0.001$ from control.

P47), malonic acid phenyl ester ethyl ester (MRC3P133) were synthetized at the Laboratory of Organic Chemistry (University of Namur) according to Michiels et al. (2001). Purity and stability were checked by thin-layer chromatography, nuclear magnetic resonance and elemental analyses. These molecules were dissolved in dimethylsulfoxide, which was serially diluted into incubation solutions or media at final concentrations not exceeding 0.15% (which corresponds to 10^{-3} M of the MRC molecules). Controls were performed in the presence of dimethyl sulfoxide at the same concentration as in the highest one for the tested molecule.

2.13. Statistical analysis

Results are presented as means ± 1 S.D. Statistical analyses were performed using Student's *t*-tests.

3. Results

3.1. Effects of the drugs on mitochondrial respiration

Among the different compounds that increase the respiratory activity by protecting complexes I and III, bilobalide is one of the most potent molecules (Janssens et al., 1999). Bilobalide is a terpenoid present in *Ginkgo biloba* leaf extract that has been shown to be protective against ischemia both in vitro (Janssens et al., 1995) and in vivo (Pietri et al., 1997; Krieglstein et al., 1995). Due to this potency and since it has a rigid structure (Table 1), it was used as template. Three molecules with two penta-lactone rings in different positions were obtained. These molecules were tested for their capacity to increase the respiratory activity as measured by RCR and only BN50504 was able to increase the RCR (Table 1). These results suggest that the two ketone groups need to be close one from each other. Seven original analogs sharing this pharmacophoric element were designed in silico and, subsequently, synthetized. They were tested on isolated mitochondria and two of them, MRC2P119 and MRC2P57 displayed a high activity (Table 1). Their properties were then further characterized.

MRC2P119 and MRC2P57 increased the RCR with a bell-shaped curve, indicating that the effect was limited at high concentrations. This was already observed for bilobalide and the other drugs previously tested (Janssens et al., 1999; Janssens et al., 2000a). The effect was maximal at 10^{-6} M for MRC2P119 and at 10^{-7} M for MRC2 P57 (Fig. 1). As observed for bilobalide, the increase in

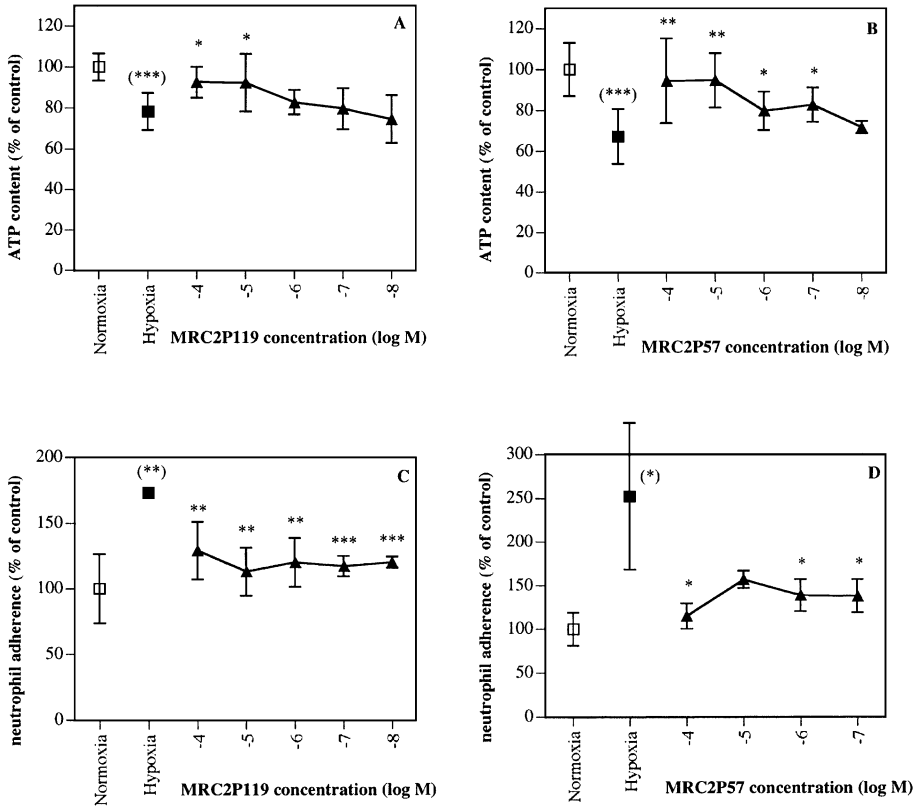


Fig. 3. Effect of MRC2P119 (A, C) and MRC2P57 (B, D) on the hypoxia-induced activation of human umbilical vein endothelial cells. Cells were incubated for 2 h in hypoxia in the presence or in the absence on different concentrations of the molecules. Control cells were incubated for 2 h in normoxic conditions. ATP was assayed in cell lysates by the luciferine-luciferase system (A, B). Neutrophil adherence was measured after the addition of labeled neutrophils for 5 min and subsequent washings (C, D). Results are expressed in percentage of normoxic condition as mean ± S.D. ($n=3$ for C and D, $n=6$ for A and B). (*), (**) or (***): $P<0.05$, $P<0.01$ or $P<0.001$ from normoxic control *, ** or ***: $P<0.05$, $P<0.01$ or $P<0.001$ from hypoxic control.

RCR was due to a decrease in state 4 respiration rate (Fig. 1).

Bilobalide is able to protect the decrease in oxygen consumption due to complex I or complex III inhibition, but not when this decrease is due to uncoupling by a protonophore (Janssens et al., 1999). Similarly, MRC2P199 and MRC2P57 prevented the decrease in oxygen consumption due to complex I inhibition by amytal with 30% protection and 38% protection, respectively, at 10^{-6} M (Fig. 2A and B) as well as the decrease in oxygen consumption due to complex III inhibition by myxothiazol with 22% protection and 25% protection, respectively, at 10^{-6} M (Fig. 2C and D). On the other hand, none of the molecules could prevent the inhibition of oxygen consumption due to the uncoupling induced by *m*-CCP (Table 2). These results suggest a specificity of both molecules towards the same biochemical target(s) identified for bilobalide.

In order to confirm these effects, the activity of complex I was assayed. Table 3 shows that MRC2P119 and MRC2P57 did significantly increase complex I activity. The effect was obtained at lower concentrations of MRC2P17 than of MRC2P119, as already been observed for the increase in RCR.

3.2. Effects of the drugs on cells incubated in hypoxia

Hypoxic conditions leads to a loss of the energetic molecules within the cells and eventually to cell death. Moreover, in endothelial cells, the decrease in ATP content leads to cell activation, with the release of inflammatory mediators and the development of an inflammatory response (Michiels et al., 2000). Since MRC2P119 and MRC2P57 increase the mitochondrial respiratory activity, we sought whether they could prevent the hypoxia-induced decrease in ATP. This decrease in ATP in cells exposed to hypoxia results from a slow down of the respiration due to oxygen deficiency.

Two hours of hypoxia incubation decreased by 25–35% the ATP content in endothelial cells. MRC2P119 and MRC2P57 inhibited this decrease in a concentration-dependent way (Fig. 3A and B). The effect was maximal at 10^{-4} M for MRC2P119 (66% protection) and MRC2P57 (83% protection). As previously shown, incubation of endothelial cells in hypoxia leads to their activation as evidenced by an

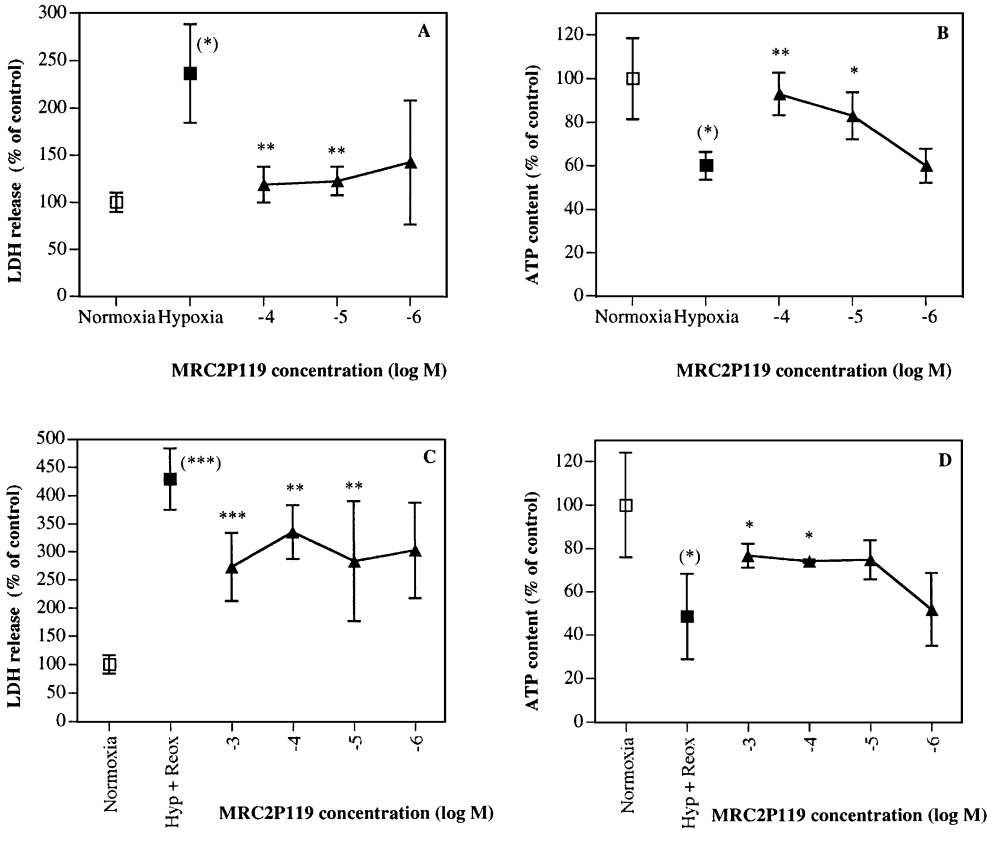


Fig. 4. Effect of MRC2P119 on hypoxia or hypoxia-reoxygenation effects on SH-SY5Y neuroblastoma cells. Cells were incubated for 24 h in hypoxia in the presence or in the absence of different concentrations of MRC2P119 (A, B). Afterwards, they were reoxygenated for 24 h (C, D). Control cells were incubated for 24 or 24 + 24 h in normoxic conditions. LDH activity was assayed in the medium (A, C). ATP concentration was assayed in cell lysates by the luciferine-luciferase system (B, D). Results are expressed in percentage of control as means ± S.D. ($n = 3$ for A, B and D, $n = 6$ for C). (*) or (***): $P < 0.05$ or $P < 0.001$ from normoxic control. *, ** or ***: $P < 0.05$, $P < 0.01$ or $P < 0.001$ from hypoxic control.

increase in their adhesiveness for neutrophils (Arnould et al., 1993; Fig. 3C and D). This process is not due to cell death since viability is maintained higher than 95% (Arnould et al., 1993; data not shown). MRC2P119 and MRC2P57 inhibited this increase with maximal effect at 10^{-5} M for MRC2P119 (82% protection) and at 10^{-4} M for MRC2P57 (90% protection).

If prolonged, hypoxia leads to cell death. According to the duration of hypoxia, reoxygenation allow cells to recover, but can also lead to further cell death. When incubated 24 h under hypoxia, the ATP content of SH-5YSY neuroblastoma cells decreased by 40% and MRC2P119 prevented this decrease (Fig. 4B). This effect was concentration-dependent and maximal at 10^{-4} M (83% protection). In parallel, a loss of viability is observed as evidenced by the increase in LDH release, which was also inhibited by MRC2P119 (Fig. 4A). When the cells were reoxygenated for 24 h after being incubated in hypoxia for 24 h, a further increase in cell mortality was observed in parallel with a further decrease in ATP content (Fig. 4C and D). MRC2P119 when present during both incubations led to higher ATP content and lower mortality.

4. Discussion

Anti-ischemic therapies often act to correct the balance between organ oxygen supply and demand by increasing blood flow or reducing oxygen requirements (Cohn, 1998; Savonitto and Ardissino, 1998). This is especially true for myocardium where medications include nitrates, beta-blockers as well as antihypertensive drugs like calcium channel blockers (Tzivoni, 2001) or angiotensin-converting enzyme inhibitors (Pitt, 2000). Anti-thrombotic therapy is also recommended for heart as well as cerebral ischemia. Moreover, in numerous animal models, antioxidants to protect from alterations induced by reactive oxygen species during the reperfusion period were shown to be useful (Cuzzocrea et al., 2001). However, none of these approaches actually target the cells which are undergoing the ischemic conditions. To be able to do so in every cell type would protect cells and, hence, tissues from loss of function and viability when ischemic period occurs.

Mitochondria are present in all cell types and insure the maintenance of cellular energy metabolism. Mitochondria dysfunction plays a critical role in the induction of cell death during ischemia (Borutaite et al., 1995): not only the decrease in respiratory activity is involved, but also the regulation of calcium homeostasis, the generation of reactive oxygen species and the release of apoptogenic proteins (Fiskum, 2000; Di Lisa and Bernardi, 1998). We have previously showed that drugs commonly used in the treatment of venous disease display anti-ischemic properties because they protect some of the respiratory chain components when inhibited chemically or during oxygen deficiency, thus preserving the ATP content of cells exposed to hypoxia (Janssens et al., 2000a). Among these compounds, bilobalide, one of the active compounds of the *G. biloba* leaf extract, was selected. Indeed, protective effects of the *G. biloba* extract have been demonstrated in rats against deterioration of metabolism and function caused by hypoxia in brain (LePoncin-Lafitte et al., 1980; Spinnewyn et al., 1995) and in heart (Pietri et al., 1997). This protective effect has been attributed to the terpenoid fraction of *G. biloba* extract and, at least in part, to bilobalide. The anti-ischemic activity of *G. biloba* extract has also been observed in double blind clinical studies (for a review, Yoshikawa et al., 1999), for example, in the treatment of peripheral arterial occlusive disease (Mouren et al., 1994; Blume et al., 1996) and in old patients with cerebrovascular insufficiency (Hopfenmuller, 1994). Since bilobalide is characterized by a rigid 3D structure containing three gamma-lactones, we hypothesized that these moieties were part of the chemical features required for the activity toward the mitochondria respiratory chain. We investigated three other molecules bearing two lactones in different orientations (1,5-dioxospiro-2,6-nonanedione, 2,6-dimethyl-hexahydro-furo-furan-4,8-dione, BN50504) and observed that the activity is only conserved when these lactones were adjacent to form a malonyl moiety. Based on these considerations, seven novel molecules were then designed, synthesized and tested. Two of them, MRC2P119 and MRC2P57 were able to markedly increase the respiratory activity as measured by RCR. Unsaturation of one of the cyclopentyl groups and rigidity given by a penta-ring seem to be the common features of the active molecules.

MRC2P119 and MRC2P57 showed a markedly increased RCR, which was due to a decrease in state 4 of the respiration. A dose-dependent effect was observed. State 4 reflects the proton leak across the inner mitochondrial membrane. At least two processes can influence the level of such a leak. First, state 4 mainly depends on the optimal activity and integrity of the respiratory chain namely of the different electron transporters (complexes I, III and IV) which extrude protons from the mitochondria matrix toward the intermembrane space, thus generating the proton gradient. Second, specialized proteins, uncoupling proteins (UCP) may play a role in mediating proton leak. While UCP1 is only expressed in mitochondria from brown adipose tissue, UCP2 and 3 are ubiquitously expressed. UCP1 diverts energy from ATP synthesis to thermogenesis by catalyzing a regulated leak of protons across the inner mitochondrial membrane. On the other hand, the function of UCP2 and 3 is still debated (Klingenberg and Echtay, 2001; Stuart et al., 2001). Superoxide anion has been demonstrated to increase mitochondria proton conductance and mild uncoupling decrease the mitochondria production of reactive oxygen species. This may represent a regulated feedback protective response to overproduction of reactive oxygen species (Echtay et al., 2002). MRC2P119 and MRC2P57 do not directly scavenge reactive oxygen species (data not shown), effect on UCP through this mechanism is thus unlikely. Finally, when cytosolic calcium concentration rises and/or when mitochondria are slightly

damaged, calcium cycling takes place, dissipating the proton gradient and increasing state 4. This could be prevented by calcium chelators such as EGTA (Richter and Frei, 1988). Previous results show that the effect of bilobalide in the presence of EGTA was the same as in the absence of EGTA, thus excluding a role for bilobalide as a calcium chelator or as an inhibitor of calcium cycling (Janssens et al., 1999). On the other hand, bilobalide as well as MRC2P119 and MRC2P57 were able to protect the respiratory chain from inhibition of the complex I and III activity by amytal and myxothiazol, respectively. MRC2P119 and MRC2P57 were also able to increase the activity of complex I of liver mitochondria.

Previous work defined the mechanism whereby bilobalide maintains mitochondrial coupling. Mitochondria isolated from bilobalide-treated (8 mg/kg) rats as well as control mitochondria preincubated 1 h with 0.8 μM bilobalide showed a markedly increased RCR, which was due to a decrease in state 4 of the respiration. Bilobalide was able to protect the respiratory chain toward inhibition of the complex I activity by amytal as well of the complex III activity by antimycin A or myxothiazol. Bilobalide treatment was also able to increase the activity of complex I of liver mitochondria. These results show that bilobalide increases the activity of complex I and protects both complex I and complex III from inhibition and, hence, leads to a decrease in state 4. Interestingly, bilobalide have also been shown to be protective against cerebral edema induced by triethyltin, which uncouples oxidative phosphorylation (Otani et al., 1986).

It must be noted that bilobalide also slightly decreased state 3 in native mitochondria. State 3 in optimal conditions is mainly limited by the activity of adenine translocase (Duan and Karmazyn, 1989). However, bilobalide does not affect adenine translocase activity. Succinate transport is also important for state 3 activity (Hansford, 1980, Lanau and Schoolwerth, 1979), but bilobalide did not seem to influence this parameter since a similar effect of bilobalide on state 3 was observed when succinate or glutamate/malate were used as substrates (Janssens et al., 1999). The most important effect of bilobalide in optimal conditions for mitochondria is to decrease state 4. The P/O ratio is not affected by bilobalide treatment of preincubation, which means that the same amount of O_2 is needed with or without biloblide to produce a given amount of ATP. If the mitochondria are better coupled in the presence of bilobalide (state 4 is decreased), a high rate state 3 may not be needed to obtain the same efficiency concerning ATP regeneration. This could explain why state 3 is decreased in the presence of bilobalide (Janssens et al., 1999).

Most interestingly, bilobalide totally protects the ischemia-induced decrease in RCR as well as the ischemia-induced decrease in complex I activity. Ischemia decreases state 3 and bilobalide completely prevented this decrease (Janssens et al., 2000b). The question is why a protection of state 3 is observed for ischemic mitochondria in the presence of bilobalide, while there was no protection in normal mitochondria. The following explanation is proposed: state 3 determination is mainly limited by adenine translocase activity in normal mitochondria, while complexes I and III work at around 70% of their maximal activity. However, when there are some alterations of the mitochondrial chain, the activity of the different complexes begins to be the limiting factor so that state 3 decreases and RCR is lower. Since bilobalide acts on complexes I and III, but not on adenine translocase, one can easily understand that no effect of bilobalide can be observed on state 3 when the respiratory chain optimally works. On the other hand, when there are small alterations, then the activity of the complexes is limiting and the effect of bilobalide that increases the activity of such complexes can then be observed on state 3. The effect of bilobalide is even stronger when state 3 is decreased to a larger extent, for example, after ischemia. MRC2P119 and MRC2P57 similarly increase RCR through a decrease in state 4 respiration rate and protect complex I and complex III from chemical inhibition. We propose that they act by the same mechanism as bilobalide.

Inhibition of mitochondrial respiration is one of the earliest events occurring in ischemic tissue. It was, thus, interesting to investigate whether these molecules would be beneficial for cells undergoing oxygen deficiency. Two different in vitro models were used: hypoxia-induced endothelial cell activation and hypoxia-induced loss of viability in neuroblastoma cells.

First, the pathological activation of endothelial cells by ischemic conditions has been shown to play a key role in the development of inflammatory conditions in ischemic organs (Michiels et al., 2000). The initial event of the pathological activation of endothelial cells by hypoxia in vitro is a decrease in intracellular ATP content, which leads to an increase in cytosolic calcium concentration. This decrease is due to a decrease in the mitochondrial respiration. Calcium can then induce the activation of phospholipase A_2, responsible for an increase in the synthesis of prostaglandins and platelet-activating factor (Michiels et al., 2000). This process results in an increase in endothelial cell adhesiveness for neutrophils (Arnould et al., 1993) and to the subsequent activation of these blood cells (Arnould et al., 1994). Hypoxia is, thus, able to initiate, via an active role of the endothelium, an inflammatory response, which then initiates and promotes tissue damages. According to this process, preservation of ATP regeneration by drugs should prevent the hypoxia-induced endothelium activation and, hence, would protect tissue from subsequent alteration. When present during the hypoxia incubation, MRC2P119 and MRC2P57 were able to prevent the hypoxia-induced decrease in ATP content in endothelial cells. Moreover, the subsequent increase in the adhesiveness of endothelial cells for neutrophils was also inhibited. These results indicate that these molecules could be useful to prevent endothelial cell activation and tissue damage in ischemic conditions.

Second, prolonged ischemic exposure eventually leads to cell death. Neuronal cells are particularly sensitive to such

conditions, due to their high demand for high energy nucleotide derivatives. Indeed, 24 h hypoxia leads to a 40% decrease in ATP content of SH-5YSY neuroblastoma cells with concomitant loss of viability. MRC2P119 at 10^{-4} M completely prevented both the decrease in ATP and the cell mortality, indicating that preserving ATP is beneficial for cell outcome.

The only way to prevent ischemic tissue necrosis is to restore blood flow. However, reperfusion is associated with further damages (Carden and Granger, 2000). This can be reproduced in vitro (Fig. 4C): reoxygenation of SH-5YSY cells after 24 h hypoxia led to delayed cell death as evidenced by increased LDH release. The reoxygenation/reperfusion injury is the consequence of events occurring at the moment of reperfusion like the release of high amounts of reactive oxygen species and, hence, antioxidants may be proven to be useful. However, this process is mainly the result of changes occurring during the period of ischemia (Ferrari, 2000; Michiels et al., 1992) and preventing these events to occur is very potent. Indeed, MRC2P119 is able to inhibit the hypoxia–reoxygenation-induced cell death, probably because it has protected the ATP content of cells during the hypoxia period.

In conclusion, targeting the mitochondrial respiratory chain whose activity is decreased in ischemic conditions is a new approach with huge therapeutic perspective: sparing of high energy phosphates, such as ATP, during ischemia would preserve cellular integrity and functions during ischemia, but would also prevent reactive oxygen species formation during reperfusion, thereby favouring functional recovery. Due to the ubiquist localisation and need for mitochondria, this approach may be useful for every pathological situation where ischemia takes place.

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