Biological Evaluation of Some Selected Cyclic Imides: Mitochondrial Effects and *in vitro* Cytotoxicity

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Cyclic imides such as succinimides, maleimides, glutarimides, phthalimides and their derivatives contain an imide ring and a general structure -CO-N(R)-CO- that confers hydrophobicity and neutral characteristic. A diversity of biological activities and pharmaceutical uses have been attributed to them, such as antibacterial, antifungal, antinociceptive, anticonvulsant, antitumor. In spite of these activities, much of their action mechanisms at molecular and cellular levels remain to be elucidated. We now show the effects of several related cyclic imides: maleimides (S2, S2.1, S2.2, S3), glutarimides (S4, S5, S6), 4-aminoantipyrine derivatives (L1, F1, AL1, F1.14, F1.2) and sulfonated succinimides (RO1, FA, FE, FD, MC, DMC) on isolated rat liver mitochondria, B16-F10 melanoma cell line, peritoneal macrophages and different bacterial streams. The effects on mitochondrial respiratory parameters, cell viability and antibacterial activity were also evaluated.

The results indicated that S3, S5 and S6 caused an increased oxygen consumption in the presence of ADP (state III) or its absence (state IV), while all other compounds decreased those parameters at different degrees of inhibition. All the compounds decreased the respiratory control coefficient (RCC). Loss of cell viability of peritoneal macrophages and the B16-F10 cell line was observed, L1 and S2.1 being more effective. S1, S2, S3, L1 and F1 compounds showed antibacterial activity at experimental concentrations.

Key words: Cyclic Imides, Mitochondrial Respiratory Chain, Cytotoxicity

Introduction

Synthetic cyclic imides, such as succinimides, maleimides, glutarimides, phthalimides and related compounds, possess structural features, which confer potential biological activity and pharmaceutical use. Their molecules contain an imide ring and the general structure -CO-N(R)-CO-, so that they are hydrophobic and neutral, and can therefore cross biological membranes *in vivo* (Haergreaves *et al.*, 1970). All of the various classes of cyclic imides have received attention due to their antibacterial, antifungal, analgesic (Cechinel Filho *et al.*, 2003) and antitumor activities (Sami *et al.*, 2000; Wang *et al.*, 2000). Some of these effects appear to be related to the size and electrophilic characteristics of substituent groups on the imide ring that can modify their steric properties (Nunes, 1986; Cechinel Filho, 1995; Lima *et al.*, 1999; López *et al.*, 2003). Beside these interesting biological effects, some cyclic imides, *e.g.*, chlorophthalim (Adomat and Börger, 2000), *N*-aryltetrahydrophthalimide (Birchfield and Casida, 1997) and *N*-(4-chloro-2-fluoro-5-propagyloxy)-phenyl-3,4,5,6-tetrahydrophthalimide (Watanabe *et al.*, 1998) are peroxidizing herbicides, a class of the herbicides that inhibited the protoporphyrinogen IX oxidase, a key enzyme of heme and chlorophyll biosynthesis (Böger and Wakabayashi, 1995).

In spite of the diversity of biological effects that have been assigned to cyclic imides, much of their biological and toxicological action mechanisms at molecular and cellular levels remain to be elucidated. In the assessment of biological properties of new compounds, several models have been used (Cadena *et al.*, 1998, 2002; Senff-Ribeiro *et al.*, 2003, 2004). We evaluated the effects of several related cyclic imide compounds: maleimides (S2, S2.1, S2.2, S3), glutarimides (S4, S5, S6), 4-aminoantipyrine derivatives (L1, F1, AL1, F1.14, F1.2) and sulfonated succinimides (RO1, FA, FE, FD,

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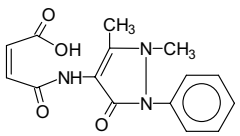
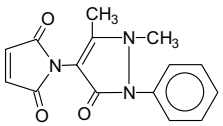
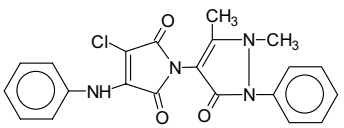
4-Aminoantipyrine Derivatives



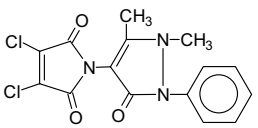
AL1



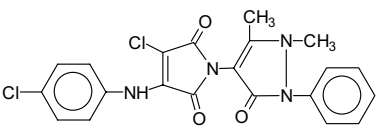
L1



F1.2

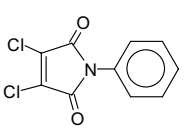


F1

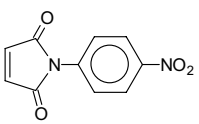


F1.14

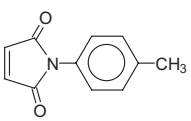
Maleimides



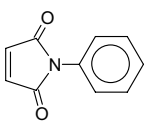
S1



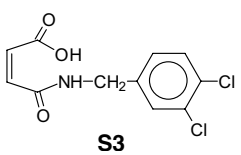
S2



S2.1

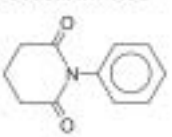


S2.2

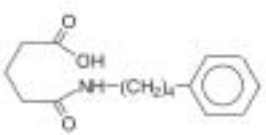


S3

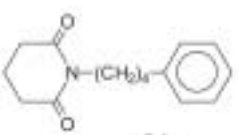
Glutarimides



S4

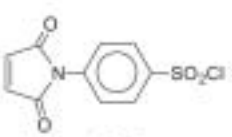


S5

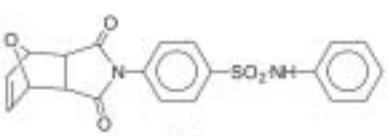


S6

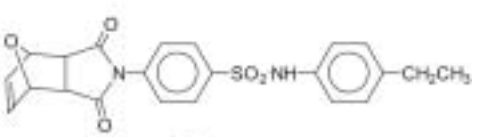
Sulfonated Succinimides



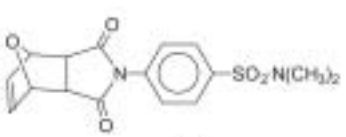
RO1



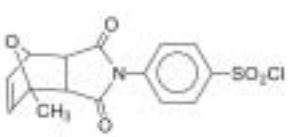
FA



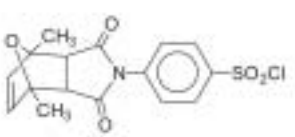
FE



FD



MC



DMC

Fig. 1. Chemical structures of cyclic imides studied: Derivatives of 4-aminoantipyrine, maleimides, glutarimides and sulfonated succinimides.

MC, DMC) (Fig. 1) on some energy-linked functions in isolated rat liver mitochondria and their cytotoxicity on B16-F10 melanoma cell line and peritoneal macrophages. The antibacterial activity was also evaluated against three pathogenic bacteria by a diffusion method.

Materials and Methods

Materials

Carbonyl cyanide *m*-chlorophenylhydrazone (FCCP), dimethylsulfoxide (DMSO), sodium glutamate, sodium succinate, rotenone, bovine serum albumin (BSA), *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), mannitol, sucrose, phosphoenolpyruvate (PEP), pyruvate kinase, NADH, L-glutamate dehydrogenase (GDH) and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were commercial products of highest available purity.

All the cyclic imides employed were synthesized at NIQFAR – University of Itajaí Valle, Itajaí, SC, Brazil, and their structures were confirmed by spectroscopic methods. The compounds were solubilized in DMSO and used at different concentrations, depending on the test. This solvent did not cause any effects on the experimental systems utilized.

The following cyclic imides were used, namely: a) 4-aminoantipyrine (4-AAP) derivatives: *N*-antipyrine-maleimide (L1, M_r 283.15) (Cechinel Filho *et al.*, 1998), *N*-antipyrine-maleamic acid (AL1, M_r 301.15), *N*-antipyrine-3,4-dichloromaleimide (F1, M_r 352.15) (Campos *et al.*, 2002), *N*-antipyrine-3-chloro-4-aniline-maleimide (F1.2, M_r 408.71), and *N*-antipyrine-3-chloro-4(4-chloro)-aniline-maleimide (F1.14, M_r 443.21) (De Campos, 2001); b) maleimides: 4-nitro-*N*-phenyl-maleimide (S2, M_r 218.16), 4-methyl-*N*-phenyl-maleimide (S2.1, M_r 187.19) (Lima *et al.*, 1999), *N*-phenyl-maleimide (S2.2, M_r 172.15) (Chechinel Filho *et al.*, 1996; Andricopulo *et al.*, 1999), and *N*-methyl-3,4-dichloro-phenyl-maleimidic acid (S3, M_r 237.16) (Cechinel Filho, 1995); c) glutarimides: *N*-aryl-glutarimide (S4, M_r 189.11), *N*-butyl-phenyl-glutarimide (S6, M_r 245.15), and *N*-butyl-phenyl-glutamic acid (S5, M_r = 262.15) (Stiz *et al.*, 2000), and d) sulfonated succinimide derivatives: *N*-(*p*-chlorosulfonylphenyl)-maleimide (RO1, M_r 271.50), *N*-(*p*-*N*′-phenylsulfamoylphenyl)-8-oxabicyclo[2.2.1]-hept-4-en[5,6-*c*]-succinimide (FA, M_r 396.27), *N*-(*p*-*N*′-4-ethylphenylsulfamoyl)-8-oxabicyclo[2.2.1]-hept-4-en[5,6-*c*]-succinimide (FE, M_r 424.24), *N*-(*p*-*N*′,*N*′-dimethylsulfamoylphenyl)-8-oxabicyclo[2.2.1]-hept-4-en[5,6-*c*]-succinimide (FD, M_r 348.19), *N*-(*p*-chlorosulfonyl)-8-oxabicyclo[2.2.1]-hept-4-en-3-methyl[5,6-*c*]-succinimide (MC, M_r 353.67), and *N*-(*p*-chlorosulfonyl)-8-oxabicyclo[2.2.1]-hept-4-en-3,6-dimethyl[5,6-*c*]-succinimide (DMC, M_r 367.68) (López *et al.*, 2003).

Animals

Male albino rats (Wistar strain, 220–300 g) were used on mitochondrial evaluation experiments and male albino Swiss mice (6–8 week-old) were used for macrophages collection. They were housed at 22 ± 1 °C under a 12:12 h light-dark cycle (lights on at 7:00 a.m.) and had free access to a standard laboratory diet (Purina®) and water *ad libitum.* All animals were starved 12 h before being sacrificed. All recommendations of Brazilian National Law (number 6638, 05. Nov. 1979) for Scientific Management of animals were respected.

Preparation of rat liver mitochondria

Mitochondria were isolated by differential centrifugation according to Voss *et al.* (1961), using an extraction medium consisting of 250 mM mannitol, 10 mM HEPES-KOH, pH 7.2, 1 mM ethylene glycol-bis(β-amino-ethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and 0.1% BSA. Only mitochondrial preparations with a respiratory control (RC) above 4.0 were used. Each compound was evaluated by 2 min incubation with the mitochondrial preparations before initiation of assays. For each assay to be valid, duplicate solvent controls with DMSO were included.

Oxygen uptake

Oxygen uptake and oxidative phosphorylation measurements were carried out in a 1.3-ml water-jacketed closed chamber with magnetic stirring at 28 °C. Oxygen uptake was measured polarographically using a Clark oxygen electrode linked to a Gilson oxygraph (Chance and Williams, 1955). The standard respiratory medium contained 125 mM D-mannitol, 65 mM KCl, 10 mM HEPES-KOH, pH 7.2, 0.1 mM EGTA and 0.1% BSA. The medium was supplemented with 0.8 mM KH_2PO_4, 0.08 mM ADP, 5 mM glutamate, 0.16 mM ADP and 2 mg of mitochondrial protein. Each compound under study was added to the medium 2 min before initiating the reaction with glutamate. After recording the stable basal respiration, state III respiration was induced by addition of ADP. The respiratory rate is

expressed as nmol of oxygen consumed min^{-1} mg^{-1} of mitochondrial protein (Estabrook, 1967).

Cell line and culture conditions

The B16-F10 mouse melanoma cell line was kindly provided by the Ludwig Institute for Cancer Research (São Paulo, Brazil). It was maintained in liquid nitrogen under a low number of passages. After thawing, the cells were grown in monolayer cultures in MEM containing 7.5% fetal bovine serum (FBS), penicillin (100 units/ml) and gentamycin (50 μg/ml). The cultures were kept at 37 °C in a humidified atmosphere plus 5% CO_2. Release of cells was performed by treatment for a few minutes with a trypsin solution (0.2%). After counting, cells were then resuspended in an adequate volume of MEM supplemented with 7.5% FBS and again plated in the presence or absence of the compounds under study.

Peritoneal macrophage preparation

Peritoneal macrophages were collected by infusion the peritoneal cavity with 10 ml of ice-cold sterile PBS. After centrifugation, the cell pellet was washed twice with cold, sterile PBS and then resuspended in the same solution. The cells were counted using a Neubauer hemocytometer and were plated so as to adhere onto a tissue culture plate. After 30 min at 37 °C under a 5% CO_2 atmosphere, the cells were washed twice with warm sterile PBS to remove nonadherent cells. Adherent macrophages were then incubated in a standard medium consisting of MEM supplemented with 10% FBS, 100 U/ml penicillin and 50 μg/ml gentamycin. Accordingly, more than 90% of adherent cells were macrophages (Adams, 1979; Moretão *et al.*, 2003) and the preparation was not further purified.

Cell viability

Viability assays were carried out on 96-well plates (TPP-multi well plates, Trasadingen, Switzerland). B16-F10 cells or peritoneal macrophages (5×10^5 cells/well) were plated and allowed to adhere for 2 h before incubation with the drugs. The drugs, dissolved in medium culture (MEM), were added at varying concentrations (0.08 to 0.32 mM for macrophages or 0.2 to 1.0 mM for B16-F10 cells) and 3 h thereafter, the supernatants were removed and the cells were washed with PBS. The cell number on each well was determined using the MTT method. MTT was dissolved in Hank's balanced saline solution (HBSS) at 5 mg/ml. The MTT solution (20 μl) was then added to each well and the plates were incubated at 37 °C for 3 h. The MTT solution was removed and DMSO was added and mixed thoroughly to dissolve the dark blue crystals. Absorbance was then determined using a MicroELISA Reader (Bio-Rad, Madison, Georgia, USA) at 550 nm for the sample and 655 nm for the reference. Results are expressed as the cell number, which was determined using a standard curve of number of cells against absorbance. In control experiments, the MEM contained equivalent amounts of vehicle. The cell viability of the controls was normalized to 100%.

Assay for antibacterial activity: diffusion method

The compounds were investigated against *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923) and *Staphylococcus epidermidis* (ATCC 12228), which were obtained from the Microbiology Laboratory of Catholic Pontifice University, Curitiba, PR, Brazil, from Laborclin.

The diffusion method was used as described by Bauer *et al.* (1966). Bacteria were previously activated in brain heart infusion (BHI) and 0.15 ml of the inoculum, with a turbidity equivalent to that of 0.5 McFarland standard, was inoculated by confluent swabbing on Muller-Hinton agar plates. Disks impregnated with DMSO solutions of the compounds under study (250 and 500 μg/disk) were placed on the surface of precultured agar. The plates were then incubated for 24 h at 37 °C and inhibitory zones were recorded. Tetracycline (30 μg), ampicillin (10 μg) and cyprofloxacin (5 μg) were used as controls.

Protein determination and statistics

The mitochondrial protein was assayed by the method of Lowry *et al.* (1951) calibrated with bovine serum albumin. Statistical analysis of the data were carried out using analysis of variance (ANOVA) and the Tukey test for average comparison. Mean ± SD values were used. Significance was defined as $P < 0.05$.

Results

Effect of cyclic imides on mitochondrial parameters

Fig. 2 to 5 show the effects of cyclic imides (46 μM) on functionally intact mitochondria, which oxidized the glutamate substrate. The parameters

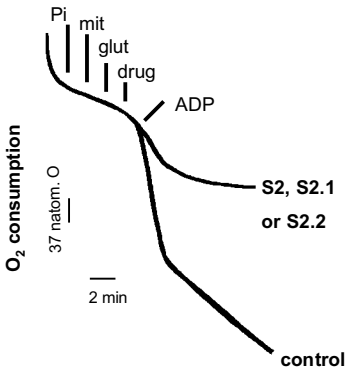


Fig. 2. Effects of maleimides on mitochondrial respiration with sodium glutamate as substrate; representative trace of oxygen consumption using S2, S2.1 and S2.2. Conditions for oxygen uptake measurements are described in the Materials and Methods section. State III, mitochondrial oxygen uptake in the presence of ADP and substrate. 100% activity corresponds to 139.45 ± 12.1 nmol of oxygen consumed per min per mg of mitochondrial protein. State IV, mitochondrial oxygen uptake after exhaustion of ADP. 100% activity corresponds to 25 ± 2.8 nmol of oxygen consumed per min per mg of protein. RCC, respiratory control coefficient. 100% corresponds to 5.6 ± 0.5. Each value represents the mean ± SD of four different experiments. *Significantly different from control ($P < 0.05$).

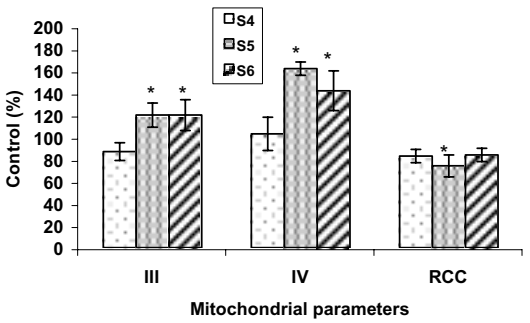


Fig. 3. Effects of glutarimides on mitochondrial respiration with sodium glutamate as substrate. Conditions for oxygen uptake measurements are described in the Materials and Methods section. State III, mitochondrial oxygen uptake in the presence of ADP and substrate. 100% activity corresponds to 139.45 ± 12.1 nmol of oxygen consumed per min per mg of mitochondrial protein. State IV, mitochondrial oxygen uptake after exhaustion of ADP. 100% activity corresponds to 25 ± 2.8 nmol of oxygen consumed per min per mg of protein. RCC, respiratory control coefficient. 100% corresponds to 5.6 ± 0.5. Each value represents the mean ± SD of four different experiments. *Significantly different from control ($P < 0.05$).

under analysis were the rate of oxygen consumption in the presence of ADP (state III) and after consumption of ADP (state IV) and the respiratory control coefficient (RCC). Fig. 2 shows representative traces of oxygen consumption by mitochondria in the presence of the maleimides S2, S2.1, S2.2. Strong inhibition of state III suggests effects on the electron transfer respiratory chain. In contrast, S3 maleimide stimulated both state III and IV (14 and 60%) while it decreased RCC by 29%.

Both S5 and S6 glutarimides promoted a discrete stimulation of state III (20%) and an accentuated stimulatory effect on state IV by 62 and 42%, respectively. S4 caused a discrete inhibition of state III (13%) (Fig. 3), state IV not being affected. As a consequence of the decrease of state III and the increased rate of O_2 consumption during state IV, RCC was also affected by the cyclic imides under analysis. The glutarimides S4, S5 and S6 lowered RCC by 29, 26, and 16%, respectively (Fig. 3).

The effects of 4-AAP derivatives (L1, F1, AL1, F1.14, and F1.2) on mitochondrial oxygen consumption are shown in Fig. 4. L1 and F1 caused an accentuated decrease of O_2 consumption by liver mitochondria (Fig. 4A). AL1, F1.14, and F1.2 inhibited state III by 22, 41, 22%, respectively, and caused a discrete effect on state IV (Fig. 4B). These compounds decreased RCC by 22, 32, and 17%, respectively.

Sulfonated succinimides (FA, FE, FD, RO1, MC and DMC) caused an inhibition of state III, by 68, 73, 43, 15, 29, and 13%, respectively (Fig. 5). State IV was also inhibited by 25, 16, and 12% for FA, MC, and FE, respectively, while FD stimulated state IV by 40%. DMC and RO1 did not affected this parameter. RCC was decreased by FA, FE, and FD by 57, 69, and 59%, respectively.

The results obtained with mitochondria motivated an investigation with other experimental models. Therefore, antibacterial effects were evaluated in addition to cell viability of peritoneal macrophages and B16-F10 melanoma.

Antibacterial activity

The data from Table I show the antibacterial activity of cyclic imides, as tested by an agar diffusion method using gram negative and gram positive bacteria strains. Antibiotics specific for each one (tetracycline for gram positive *S. aureus* and *S. epidermidis*; ampicillin and cyprofloxacin for gram negative *E. coli*) were used as positive controls. It was observed that only the maleimide S2 and the 4-AAP derivative L1 inhibited the bacterial

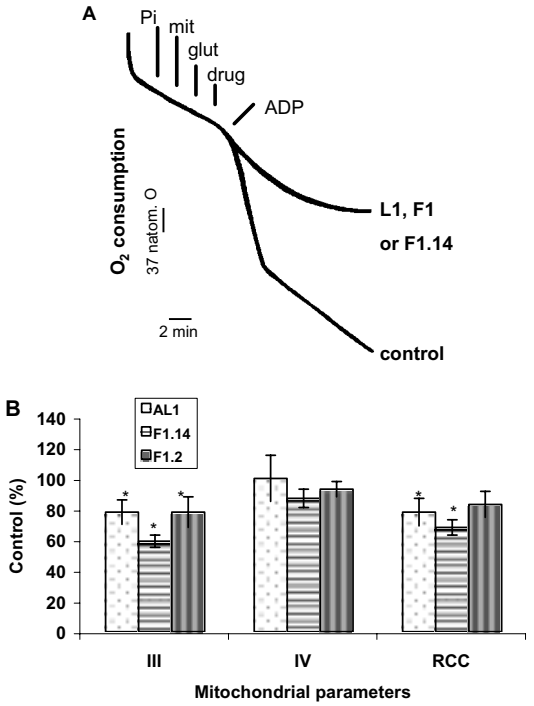


Fig. 4. Effects of 4-aminoantipyrine derivatives on mitochondrial respiration with sodium glutamate as substrate. A. Representative trace of oxygen consumption using L1 and F1. B. Effects of AL1, F1.2 and F1.14. Conditions for oxygen uptake measurements are described in the Materials and Methods section. State III, mitochodrial oxygen uptake in the presence of ADP and substrate. 100% activity corresponds to 139.45 ± 12.1 nmol of oxygen consumed per min per mg of mitochondrial protein. State IV, mitochondrial oxygen uptake after exhaustion of ADP. 100% activity corresponds to 25 ± 2.8 nmol of oxygen consumed per min per mg of protein. RCC, respiratory control coefficient. 100% corresponds to 5.6 ± 0.5. Each value represents the mean ± SD of four different experiments. * Significantly different from control ($P < 0.05$).

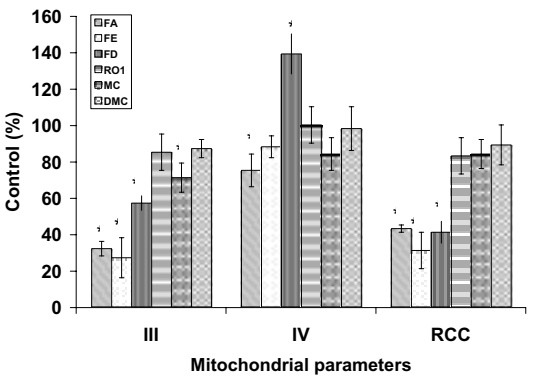


Fig. 5. Effects of sulfonated succinimides on mitochondrial respiration with sodium glutamate as substrate. Conditions for oxygen uptake measurements are described in the Materials and Methods section. State III, mitochondrial oxygen uptake in the presence of ADP and substrate. 100% activity corresponds to 139.45 ± 12.1 nmol of oxygen consumed per min per mg of mitochondrial protein. State IV, mitochondrial oxygen uptake after exhaustion of ADP. 100% activity corresponds to 25 ± 2.8 nmol of oxygen consumed per min per mg of protein. RCC, respiratory control coefficient. 100% corresponds to 5.6 ± 0.5. Each value represents the mean ± SD of four different experiments. * Significantly different from control ($P < 0.05$).

growth of all tested species, the effect occurring to a lower degree than for the antibiotic proportion controls. The maleimide S3 and the 4-AAP derivative F1 inhibited the growth of *S. aureus*.

In vitro effect on peritoneal macrophage viability

Fig. 6 shows that the viability of peritoneal macrophages as evaluated by the MTT assay, was also affected by cyclic imide derivatives. The 4-AAP derivative L1 was the most potent among the cyclic imides tested, causing 100% reduction of the viability at 0.08 mM. In addition, F1 caused a dose-dependent reduction of cell viability. The sulfonated succinimides MC and RO1 also reduced the machophage viability by 70 and 90%, respectively, but at a higher concentration of 0.32 mM (Fig. 6).

In vitro effect on B16-F10 viability

Fig. 7 shows that the 4-AAP derivative L1 and the maleimides S2, S2.1 and S2.2 were highly cytotoxic to B16-F10 melanoma cells. L1 at 0.2 mM, reducing the cell viability to 40%, and 90% of cell death at 0.4 mM, was showing a dose-dependent effect. Cell death was also observed with the maleimides S2.2, S2.1 and S2 at 0.2 mM, this concentration was lowering the cell viability to only 3–10%. All other tested compounds did not cause any significative effect on B16-F10 cells under the experi-

Table I. Antibacterial activity of cyclic imides. This activity was measured using the diffusion agar method. Strains of *S. aureus*, *S. epidermidis* and *E. coli* were examined. The inhibition of their growth was tested and measured in comparison with standard antibiotics. The data document the halo of inhibition diameter expressed in mm.

	Staphylococcus aureus	*Staphylococcus epidermidis*	*Escherichia coli*
Tetracycline (30 μg)	25	16	n.d.
Ampicillin (10 μg)	n.d.	n.d.	11
Cyprofloxacin (5 μg)	n.d.	n.d.	46
S2	12	9	8
S3	9	0	0
S4, S5, AL1	0	0	0
L1	8	10	9
F1	8	8	0
F1.14, F1.2, FA, FE, FD, MC, DMC	0	0	0

n.d. = not determined.

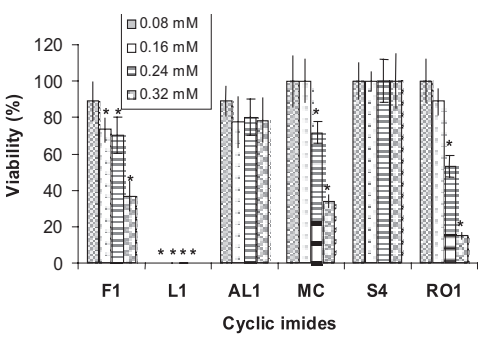


Fig. 6. Effects of cyclic imides on the viability of peritoneal macrophages. Viability was measured at different concentrations of each compound indicated using the MTT reduction assay (n = 3). Values give the mean ± SD. * $P < 0.05$.

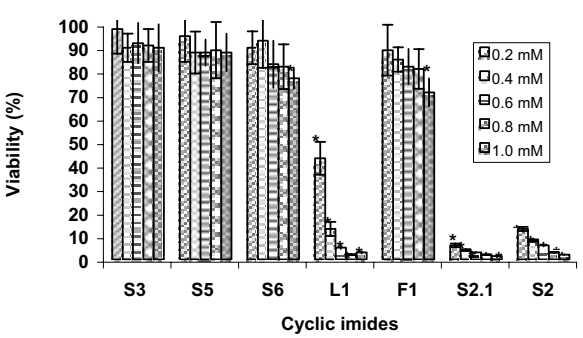


Fig. 7. Effects of cyclic imides on the viability of B16-F10 cells. This was measured at different concentrations of each compound indicated using the MTT reduction assay (n = 3). Values give the mean ± SD. * $P < 0.05$.

mental conditions. Experiments perfomed with Trypan blue gave similar results (data not shown).

Discussion

We now demonstrate that cyclic imides were active in respect to selected biological models. In contrast with the maleimide S3, which stimulated state III of the mitochondrial respiration, the maleimides S2, S2.1, S2.2 (Fig. 2A) inhibited oxygen consumption in the presence of ADP. This could be due to the integrity of the imide ring, common to this class of compounds, while S3 has an imide acid form (Fig. 1). The maleimide S3 and the glutarimides S5, S6 were able to stimulate both state III and IV (Fig. 3). The presence of a carboxyl group in S3 and S5 could explain such an effect, since the presence of a dissociable proton in their structures would confer a proton carrier capacity. However, other structural features could be important since the glutarimide S6, that promoted an accentuated stimulation of state IV, lacks a carboxyl group (Fig. 1). This compound has a high lipophilic character which could also be involved in its state IV stimulation. Concerning the glutarimide S5, the presence of a carboxyl group and a carbon chain between the imide ring and the phenyl group could explain its stimulatory effect on state IV. The absence of the *n*-butyl group in S4 could be responsible for the different effect (Fig. 3) of this compound, when compared to the other tested glutarimides. As pointed out by some authors, the size and electrophilic characteristics of substituent groups of the imide ring can be an important factor for the diversity of biological ef-

fects caused by this class of compounds (Cechinel Filho, 1995; Lima *et al.*, 1999; López *et al.*, 2003).

All the aminoantipyrine derivatives caused an inhibition of state III (Fig. 4). The greatest inhibition was caused by L1 and F1 (Fig. 4A), F1.14 being a more effective one than AL1 and F1.2 (Fig. 4B). F1.14 differs chemically from L1 and F1 due to the presence of a chlorobenzyl ring substituent of the imide ring (Fig. 1A). Thus, the chlorine substituent on the benzene ring in F1.14 could be important for the intensification of its inhibitory effect, since F1.2, that has no chlorine substituent on the benzyl group, showed only a discrete effect on the reduction of inhibition of state III (Fig. 4B). It is meaningful that, when two chlorine atoms are direct substitutents on the imide ring of F1, a reduction in the intensity of effects was observed on the B16-F10 cell viability, in comparison with L1. This compound caused a strongly inhibitory effect on oxygen consumption, as found with L1 (Fig. 4A). In contrast with the inhibition of state III for all 4-AAP derivatives, state IV was not altered by these compounds.

The sulfonated succinimides FA, FE, FD, and MC inhibited state III, the greatest effect being observed with FA, FE and FD (Fig. 5). The respiratory control (RC) was lowered by these compounds. FA inhibited state IV and FD stimulated state IV, while other compounds of this class had no effect on this parameter. Therefore, no specific structural characteristic allows us to establish a determining factor for the differences in observed effects between the sulfonated succinimides.

The reduction of oxygen comsumption observed with these cyclic imides could be related with the inhibition of the respiratory chain enzymatic complexes, restrictions of electron apport to the respiratory chain being due to inhibition of the dehydrogenase substrate, inhibition of ATP synthase, withdrawal of electrons because of an oxide-reduction reaction and/or changes of mitochondrial membrane fluidity, since all these parameters would decrease the rate of oxygen consumption in the presence of ADP (Nicholls and Ferguson, 1992). Alterations of mitochondrial parameters have been related with the action mechanism of several drugs, for instance the uncoupler effect that in some circumstances could explain the therapeutic effect of a drug (Skulachev, 1998). This way, these results prompted us to evaluate the effect of cyclic imides on different experimental cell models.

Since some cyclic imides have been described as antimicrobial agents (Haergreaves, 1970; Corrêa *et al.*, 1996; Cechinel Filho *et al.*, 2003), we have evaluated possible antibacterial effects against selected pathogens. The results indicated in Table I for S2, S3, L1 and F1 show their antibacterial activity, which inhibited bacterial growth at the concentration used. However, none of them showed a better perfomance than the positive controls.

On peritoneal macrophages, the 4-AAP derivative L1 was more cytotoxic (Fig. 6) causing 100% of cell death at a 0.08 mM concentration. This compound also decreased by ~ 90% the viability of the B16-F10 cell line at 0.4 mM (Fig. 7), but the most cytotoxic compound to this cell line was the maleimide S2.2, that drastically reduced the viability at a low concentration. This is a very important result because this cell line has a high resistance to the treatment with conventional antineoplasic agents (Senff-Ribeiro *et al.*, 2003; Vicente *et al.*, 1998).

Comparing the biological effects, it can be observed that the maleimides S2, S2.1 and S2.2, and the 4-aminoantipyrine derivatives L1 and F1 were the compounds that caused a drastic effect on cell viability, both with peritoneal macrophages and B16-F10 melanoma cells, and caused the greatest inhibition of oxygen consumption. However, the present data does not show differences between the intensity of effects on mitochondrial parameters caused by maleimides and 4-aminoantipyrine derivatives, suggesting that the imide ring is important for their biological effects, in accord with the observation of Nunes (1986). A few differences were observed between compounds of the same chemical class, the greatest being observed between L1 and F1 in terms of cytotoxicity. It must be emphasized that a parallel experiment of cell viability performed with Trypan blue gave a similar profile of effects (data not shown), indicating that a general effect on the cells was occurring besides those on mitochondria. An interesting observation is that compounds active on mitochondrial preparations, were also the more active ones on the cell models investigated.

The biological effects observed in the present study for the cyclic imides suggested promising applications. Further studies with these compounds are very important, in order to establish the mechanism of action involved in their effect, and they are in development in our laboratory.

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Adams D. O. (1979), Macrophages. Meth. Enzymol. **58**, 494–506.

Adomat C. and Böger P. (2000), Cloning, sequence, expression, and characterization of protoporphyrinogen IX oxidase from chicory. Pestic. Biochem. Physiol. **66**, 49–62.

Andricopulo A. D., Yunes R. A., Cechinel Filho V., Nunes R. J., Frazer J. W., and Cordes E. H. (1999), Development of a predictive statistical model for the analgesic activity of a family of imides. Pharmazie **54**, 698–704.

Bauer A. W., Kirby W. M. M., Serris J. C., and Turck M. (1966), Antibiotic susceptibility testing by a standardized single disc method. Am. J. Clin. Pathol. **45**, 493–496.

Birchfield N. B. and Casida J. E. (1997), Protoporphyrinogen oxidase of mouse and maize: Target site selectivity and thiol effects on peroxidizing herbicide action. Pestic. Biochem. Physiol. **57**, 36–43.

Böger P. and Wakabayashi K. (1995), Peroxidizing herbicides (I): Mechanism of action. Z. Naturforsch. **50c**, 159–166.

Cadena S. M. S. C., Carnieri E. G. S., Echevarria A., and Oliveira M. B. M. (1998), Effect of MI-D, a new mesoionic compound, on energy-linked functions of rat liver mitochondria. FEBS Lett. **440**, 46–50.

Cadena S. M. S. C., Carnieri E. G. S., Echevarria A., and Oliveira M. B. M. (2002), Interference of MI-D, a new mesoionic compound, on artificial and native membranes. Cell Biochem. Funct. **20**, 31–37.

Campos F., Corrêa R., Souza M. M., Yunes R. A., Nunes R. J., and Cechinel-Filho V. (2002), Studies on new cyclic imides obtained from aminophenazone with analgesic properties. Arzneim.-Forsch./Drug Res. **52**, 455–461.

Cechinel Filho V. (1995), Obtenção de compostos farmacologicamente ativos a partir de produtos naturais. Correlação, estrutura química-atividade biológica. Ph. D. Thesis, UFSC, Florianópolis, SC, Brazil.

Cechinel Filho V., Nunes R. J., Calixto J. B., and Yunes R. A. (1995), Inhibition of acetylcholine-mediated contraction of guinea pig ileum *in vitro* caused by phyllanthimide analogues: structure-activity relationships. Pharm. Sci. **1**, 399–401.

Cechinel Filho V., Vaz Z. R., Nunes R. J., Calixto J. B., and Yunes R. A. (1996), Antinociceptive activity of phyllanthimide analogues: structure-activity relationships. Pharm. Sci. **2**, 199–201.

Cechinel Filho V., Corrêa R., Vaz Z., Calixto J. B., Nunes R. J., Pinheiro T. R., Andricopulo A. D., and Yunes R. A. (1998), Further studies on analgesic activity of cyclic imides. Il. Farmaco **53**, 55–57.

Cechinel Filho V., de Campos F., Corrêa R., Nunes J. R., and Yunes R. A. (2003), Aspectos químicos e potencial terapêutico de imidas cíclicas: uma Revisão da Literatura. Quím. Nova **26**, 230–241.

Chance B. and Williams G. R. (1955) The respiratory enzymes in oxidative phosphorylation: kinectis of oxygen utilization. J. Biol. Chem. **217**, 383–393.

Corrêa R., Rosa P. W., Bella Cruz A., Savi A. O. S., Cechinel-Filho V., and Nunes R. J. (1996), Synthesis and antibacterial activity of citraconimides. Pharm. Sci. **2**, 353–355.

De Campos F. (2001), Síntese e atividade biológica de imidas derivadas da 4-aminoantipirina. MSc. Dissertation, UFSC, Florianópolis, SC, Brazil.

Estabrook R. W. (1967), Mitochondrial respiratory control and the polarography measurement of ADP/O ratios. Meth. Enzymol. **10**, 41–47.

Haergreaves M. K., Pritchard J. G., and Dave H. R. (1970), Cyclic carboxylic monoimides (Review). Chem. Res. **70**, 439–469.

Lima E. O., Queiroz E. F., Andricopulo A. D., Nunes J. R., Yunes R. A., Corrêa R., and Cechinel-Filho V. (1999), Evaluation of antifungal activity of *N*-aryl-maleimides and *N*-phenylalkyl-3,4-dichloro-maleimides. Bol. Soc. Chil. Quím. **44**, 185–189.

López S. N., Sortino M., Escalante A., de Campos F., Corrêa R., Cechinel Filho V., Nunes R. J., and Zacchino S. A. (2003), Antifungal properties of novel *N*- and alpha,beta-substitued succinimides against dermatophytes. Arzneim.-Forsch./Drug Res. **53**, 280–288.

Lowry O. H., Rosebrough N. J., Farr A. C., and Randal R. J. (1951), Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**, 265–275.

Moretão M. P., Buchi D. F., Gorin P. A. J., Iacomini M., and Oliveira M. B. M. (2003), Effect of an acidic heteropolysaccharide (ARAGAL) from the gum of *Andenanthera colubrina* (Angico Branco) on peritoneal macrophage functions. Immunol. Lett. **89**, 175–185.

Nicholls D. G. and Ferguson S. J. (1992), Bionenergetics 2. Academic Press Inc., San Diego, USA.

Nunes R. J. (1986), The chemistry and biological activity of cyclic imidobenzenosulphonyl derivatives. Ph. D. Thesis. The Hatfield Polytechnic.

Sami S. M., Dorr R. T., Alberts D. S., Solyom A. M., and Remers W. A. (2000), Analogues of amonafide and azonafide with novel ring systems. J. Med. Chem. **43**, 3067–3073.

Senff-Ribeiro A., Echevarria A., Silva E. F., Veiga S. S., and Oliveira M. B. M. (2003), Effect of a new 1,3,4-thiadizolium mesoionic compound (MI-D) on B16-F10 murine melanoma. Melanoma Res. **13**, 1–6.

Senff-Ribeiro A., Echevarria A., Silva E. F., Veiga S. S., and Oliveira M. B. M. (2004), Antimelanoma activity of 1,3,4-thiadizolium mesoionics: a structural-activity relationship study. Anti-cancer Drugs **15**, 269–275.

Skulachev V. P. (1998), Uncoupling: new approaches to an old problem of bioenergetics. Biochim. Biophys. Acta **1363**, 100–124.

Stiz D. S., Souza M. M., Golin V., Neto R. A. S., Corrêa R., Nunes R. J., Yunes R. A., and Cechinel-Filho V. (2000), Antinociceptive properties of *N*-aryl-glutaramic acids and *N*-aryl-glutarimides. Pharmazie **55**, 942–944.

Vicente J. R., Ortega V. V., and Jordana M. C. (1998), The effects of different antineoplasic agents and of pretreatment by modulation on three melanoma lines. Cancer **82**, 495–502.

Voss D. O., Campello A. P., and Bacila M. (1961), The respiratory chain and oxidative phosphorylation of rat brain mitochondria. Biochem. Biophys. Res. Commun. **4**, 48–51.

Wang J. J., Liu T. Y., Yin P. H., Wu C. W., Chern Y. T., and Chi C. W. (2000), Adamantyl maleimide induced changes in adhesion molecules and ROS are involved in apoptosis of human gastric cancer cells. Anticancer Res. **20**, 3067–3073.

Watanabe N., Che F., Iwano M., Takayama S., Nakano T., Yoshida S., and Isogai A. (1998), Molecular characterization of photomixotrophic tobacco cells resistant to protoporphyrinogen oxidase-inhibiting herbicides. Plant Physiol. **118**, 751–758.