Tolerability and Improved Protective Action of Idebenone-Loaded Pegylated Liposomes on Ethanol-Induced Injury in Primary Cortical Astrocytes

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ABSTRACT: The potential therapeutic advantages of the encapsulation of idebenone within pegylated liposomes were investigated *in vitro* on primary cortical astrocytes of rats. In particular, both the concentration-dependent effects and the therapeutic effectiveness toward excitotoxic injury, elicited by chronic treatment with ethanol (100 μM) for 12 days, were evaluated. The following parameters were taken into consideration to assay free or liposomally entrapped idebenone: lactic dehydrogenase release, respiratory capacity measured by tetrazolium salt conversion, glutamine synthetase, and the levels of constitutive and inducible 70-kDa heat shock proteins. To evaluate the effects on astrocytes, three different drug concentrations were used (0.5 μM, 5 μM, and 50 μM). At the highest concentration used (50 μM), a toxic effect of the free and liposomally entrapped drug was observed. Toxic effects seem to be due to a cellular membrane perturbation, as demonstrated by $^{45}Ca^{2+}$ permeation. The therapeutic effect of free or liposomally entrapped idebenone on ethanol-induced injury of primary cortical astrocytes was evaluated as a function of the drug concentration. The drug liposome formulation was much more effective than the free drug in counteracting the ethanol-induced damage in astrocytes, i.e., 10-times-lower doses of liposomally entrapped idebenone are able to provide a greater protective action than the free drug. The improved action of idebenone-loaded liposomes is probably due to the greater drug bioavailability at the cellular level.
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Keywords: liposomes; pegylation; cell culture; controlled release/delivery; phospholipids; primary cortical astrocytes; ethanol-induced injury; heat shock proteins; astrocyte idebenone bioavailability; idebenone therapeutic effectiveness

INTRODUCTION

Idebenone is a drug that is active at the level of the central nervous system (CNS), showing a protective action in various neuropathological events such as cerebral ischemia and vascular

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accidents.[1] This drug also has a great inhibitory effect on lipid peroxidation and other oxidative processes.[2] Idebenone has been demonstrated to protect against oxidative stress toxicity caused by glutathione depletion.[3] The protective action of idebenone was evaluated both on neurons and astrocytes.[4,5]

Astrocytes are able to effectively protect themselves against oxidative stress because of their high intracellular concentration of reduced glutathione and antioxidant enzymes.[6] However, the oxidative metabolic conversion of arachidonic acid into eicosanoids via the cyclooxygenase, lipoxygenase,[7] and epoxygenase pathways[8] is thought to have an important role in the cascade leading to neuronal damage and has been demonstrated to take place preferentially in astrocytes with respect to neurons.[9] Therefore, in some physiopathological conditions, astrocytes can have a role in neuronal damage by increasing the production of reactive oxygen species (ROS) and the expression of some stress-correlated genes such as inducible nitric oxide synthetase and 70-kDa heat shock proteins (HSP70).[10]

Ethanol is able to damage the CNS both directly and indirectly. In fact, the acetaldehyde (an ethanol metabolite), the phosphatidylethanol, and the free fatty acid ethyl esters produced after the administration of ethanol are responsible for neurodegenerative damage.[11] It has been demonstrated[12] that the chronic treatment of cultured astroglial cells with ethanol leads to an increased glutamate uptake. It has been suggested[13] that the combination of two effects, i.e, peroxidative processes in neurons and astroglial cells and the decrease of the levels of endogenous antioxidants, can significantly contribute in the neurodegenerative response after chronic ethanol exposure of CNS cells. The advantage of using an antioxidant agent, i.e., idebenone, to antagonize the neurodegenerative response that follows chronic ethanol exposure of astroglial cells was demonstrated.[14]

Idebenone is a highly lipophilic drug with a very low water solubility and the only marketed pharmaceutical formulation is an oral dosage form. The drug solubility and the binding with serum proteins determine a reduced absorption (influenced by food assumption) and a poor bioavailability at the level of the CNS (0.4% of the dose reaches the brain), respectively.[1]

It has been well demonstrated that idebenone can be solubilized within the bilayer structure of phospholipid vesicles,[15] allowing the formation of a drug delivery device that can be administered intravenously. This aspect is of particular importance in therapeutic approaches in which the parenteral route is required, namely for the treatment of pathologies based on neurodegenerative damage and membrane lipoperoxidation such as brain ischemia or cerebral trauma. Furthermore, liposome formulations can represent a valid device for drug delivery at the level of the CNS.[16,17]

In a previous article, advantages of using conventional liposomes as a carrier for idebenone were reported[14] but no data were reported on dose-response profiles of both free and liposome-entrapped drug. This aspect is investigated in this article and it is of great importance to evaluate the real potentiality of idebenone-loaded liposomes with respect to the free drug. In fact, the presence of both therapeutic (antioxidant action) and toxic (pro-oxidant property) effects for some antioxidant agents was reported to be dose-dependent.[18] Data reported in this article demonstrate that, also in the case of idebenone, a concentration-dependent toxic effect on cortical astrocytes is present. Furthermore, conventional liposomes previously used[14] are not suitable for *in vivo* systemic administration because of the rapid uptake by the reticuloendothelial system and hence they have a poor possibility to reach the CNS.

For these reasons and considering a possible *in vivo* use of idebenone–liposome formulations, in this study, idebenone-loaded pegylated liposomes were prepared and characterized. In fact, pegylated liposomes seem to be very effective in the delivery of drugs and in the treatment of pathologies at the level of the CNS particularly when a compromised function of the blood–brain barrier (increased permeabilization) occurs, i.e., tumors, ischemic events, viral, and/or bacterial infection,[19,20] thus achieving the extravasation of the colloidal carrier and a passive targeting of the entrapped drug. Therefore, in this study the advantages of idebenone-loaded pegylated liposomes in comparison with the free drug, in terms of formulation tolerability, biological effectiveness, dose-response curves, and cellular delivery, were investigated *in vitro* on a model of oxidative neurodegeneration based on primary cortical astrocytes damaged by chronic exposure to ethanol.

MATERIALS AND METHODS

Materials

Cholesterol (Chol), Tris, and HEPES buffer, ethylenediaminetetraacetic acid (EDTA), imidazole,

L-glutamine, adenosine 5′-triphosphate (ATP), Na_2HAsO_4, hydroxylamine, $MgCl_2$, dithiotreitol, trichloracetic acid (TCA), and L-γ-glutamil-hydroxamase were purchased from Sigma Chemical Co. (St. Louis, MO). Dulbecco's modified Eagle medium (DMEM) is a Life Technologies product (Milan, Italy). 1,2-Dipalmitoyl-*sn*-glycero-phosphocholine monohydrate (DPPC), 1,2-dipalmitoyl-*sn*-glycero-3-phospho-L-serine sodium salt (DPPS), and *N*-(carbonyl-methoxypolyethyleneglycol-2000)-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine sodium salt (MPEG-2000-DSPE) are Genzyme products (Suffolk, UK). Idebenone was kindly provided by Takeda Italia Farmaceutici S.p.A. Inorganic salts (purity >99.5%), NaCl, KH_2PO_4, and NaOH are products of BDH Laboratory Supplies (Poole, UK). $^{45}CaCl_2$ (4.3 Gbq/mmol) was purchased from Amersham International plc (Buckinghamshire, UK). Fetal calf serum is a Biochrom KG-Seromed product (Berlin, Germany). Double-distilled pyrogen-free water was used. HSP70 monoclonal antibody and secondary alkaline phosphate conjugated antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The chemiluminescent compound was from Tropix-CSPD (Milano). All other materials and solvents were of analytical grade (Carlo Erba, Milan, Italy).

Methods

Liposome Preparation

Liposomes were made up of DPPC/Chol/DPPS/MPEG-2000-DSPE (6:2:1:1 molar ratio). Various lipids were dissolved in a chloroform–methanol (8:2 v/v) mixture and then a lipid film was obtained by organic solvent evaporation. Idebenone was co-solubilized with liposome lipids. The lipid film was transformed into multilamellar vesicles by hydration with isotonic phosphate buffer (pH 7.4). The liposome suspension was submitted to a freeze-and-thaw procedure and then extruded through polycarbonate filters up to a final colloidal mean size of 50 nm.[16] The final lipid concentration was 25 mg/mL. The amount of idebenone added to the liposome formulation was 0.59 mg, corresponding to a drug/lipid molar fraction of 0.05. Liposome suspensions were purified by gel-permeation chromatography on a fine Sephadex G-50 column (50×1.5 cm) using phosphate buffer as the mobile phase. Only one peak due to liposome suspension was observed. No free drug was observed. Purified liposomes were lyophilized and then the organic material was solubilized with dichloromethane–methanol (7:3 v/v). This organic solution was analyzed by high-performance liquid chromatography.[21] Idebenone entrapped within liposomes was $99.5 \pm 1.7\%$ of the amount added. Idebenone-loaded liposome suspensions were suitably diluted as a function of the experiments.

Physicochemical Characterization of Liposomes

The mean size and the surface charge of the liposome colloidal suspension were characterized by light scattering experiments.[16] Liposome mean size was determined by photon correlation spectroscopy (PCS) (Zetamaster; Malvern Instruments Ltd., Spring Lane South, Worcs, UK). The experiments were performed using a 4.5-mW laser diode operating at 670 nm as light source. Size measurements were performed at a scattering angle of 90°. To obtain the mean diameter and polydispersity index of colloidal suspensions, a third-order cumulant fitting correlation function was performed by a Malvern PCS submicron particle analyzer. The real and imaginary refractive indexes were set at 1.59 and 0.0, respectively. The following parameters were used for experiments: medium refractive index 1.330, medium viscosity 1.0 mPa·s, and a dielectric constant of 80.4. The samples were suitably diluted with filtered water (Sartorius membrane filters 0.22 μm) to avoid multiscattering phenomena and placed in a quartz cuvette. Electrophoretic mobility and zeta potential distribution were measured with the Zetamaster particle electrophoresis analyzer set-up equipped with a 5-mW HeNe laser (633 nm). Also in this case, samples for zeta potential measurements were suitably diluted with filtered water. Zeta limits ranged from −120 to 120 V. Strobing parameters were set as follows: strobe delay −1.00, on time 200.00 ms, off time 1.00 ms. A Smoluchowsky constant F (Ka) of 1.5 was used to achieve zeta potential values from electrophoretic mobility.

Astrocyte Culture Preparation

Astrocyte cultures were used to evaluate both the biological effectiveness and the toxic effect of free or liposomally entrapped idebenone. Primary cortical astrocyte cultures were prepared by using cerebral cortex of 48-h-old Wistar rats. The animals were decapitated, and the cerebral cortex was separated from the meninges to avoid macrophage contamination. Astrocyte cells were obtained from the removed cerebral cortex

mechanically dissociated, centrifuged, and washed. The cells were seeded in DMEM/F12 medium containing 10% fetal calf serum, 1 mM L-glutamine, and antibiotics and incubated at 37°C in a humidified 5% CO_2/95% atmosphere. Typically, astrocytes reached confluence approximately 7–10 days after seeding. After 12 days, the experiments were performed. In fact, after this time, astrocytes are mature and completely differentiated.[22] To assess the astroglial nature of the cells, a test for glial fibrillary acidic protein (Kit SIH908; Sigma Diagnostic, St. Louis, MO) was performed before the beginning of the treatments. We only used the cultures in which immunostaining for glial fibrillary acidic protein showed that 90–95% of cells were astrocytes. The effect of free or liposome-associated idebenone on astrocytes was evaluated by incubation for 20 h.

Ethanol-Induced Damage in Astrocytes

Oxidative stress was induced in primary cortical astrocyte cultures by incubation with ethanol at a 100 μM concentration. Ethanol was added to the astrocyte cultures 2 days after the beginning of the seeding and thereafter every time the medium was changed. In addition, ethanol (10% of the initial concentration used) was added daily to astrocyte cultures to replace the amount lost by evaporation. After 12 days of ethanol treatment, astrocytes were submitted to 24 h of treatment with free or liposome-associated idebenone and then to the programmed analysis. The free drug was dissolved in dimethyl sulfoxide and then dispersed in the astrocyte culture medium. The presence of the free or liposome-entrapped idebenone did not influence the lactic dehydrogenase (LDH) assay.

LDH Release

The enzymatic activity of LDH was measured spectrophotometrically both in the culture medium and in the cellular lysate at λ_{max} 340 nm, by analyzing the nicotinamide adenine dinucleotide reduction during the pyruvate-lactate transformation.[23] To determine cellular LDH activity, the untreated (control) and treated cells were harvested, washed in phosphate-buffered saline (PBS), added to lysis buffer [Tris-HCl 50 mM (pH 7.4) + 20 mM EDTA + 0.5% w/v sodium dodecyl sulfate], submitted to sonication, and then centrifuged at 10,000*g* for 15 min. Lysed astrocyte cells were harvested and supernatants were analyzed for protein content and for LDH enzymatic activity measurement. The mixture (1 mL final volume) for LDH enzymatic analysis was obtained by mixing 33 μL of a sample with 48 mM PBS (pH 7.5) containing 1 mM pyruvate and 0.2 mM reduced pyridinic coenzyme. The release of LDH into the medium (reflecting cytotoxicity) was calculated as a percentage of the total amount, which is obtained from the sum of the LDH enzymatic activity present in the cellular lysate and that of the culture medium.[23]

Tetrazolium Salt Test (MTT)

Cell growth was monitored by the MTT (5 mg/mL), which was added to the astrocyte cultures and then incubated for 24 h. The concentration of the colored product formazan, coming from the cellular conversion of tetrazolium salts, was determined at 570 nm with a Titertek Multiskan microplate spectrophotometer (Flow Laboratories).[24] The amount of formazan is directly proportional to the number of living cells.

Determination of HSP70

For each sample, proteins (15 μg) were electrophoretically analyzed by a monodimensional sodium dodecyl sulfate–polyacrylamide (8% w/v) gel[25] using a Bio-Rad miniprotean apparatus. The electrophoretic experiments were performed at 30 mA. The gel was transferred to a nylon membrane by a semidry method at 0.65 mA/cm^2 for 90 min at room temperature. Western blots were hybridized with a mouse anti-HSP70 monoclonal antibody, which is able to recognize both the constitutive and the inducible forms of this protein family. The antibody was used at a dilution of 1:3000 and incubated overnight at 4°C. Membranes were washed and incubated first with a secondary anti-mouse immunoglobulin antibody (at a dilution of 1:2000) linked with the alkaline phosphatase, and then with a chemiluminescent substrate (Tropix-CSPD). The chemiluminescence was revealed by autoradiographic films. The amount of HSP70 was determined by assaying the films with a laser densitometer (Ultrascan XL; LKB, Milano). HSP70 values are expressed as arbitrary densitometric units with regard to the signal intensity.

Glutamine Synthetase (GS) Determination

Astrocytes were harvested, washed with PBS, and homogenized with 50 mM imidazole buffer containing EDTA 0.5 mM. The homogenate was

centrifuged at 20,000*g* for 45 min. The supernatant was used for the determination of the GS activity.[26] The incubation mixture (1.5 mL) was made up of 50 mM imidazole buffer (pH 7.2), 110 mM L-glutamine, 0.45 mM ATP, 3 mM Na_2HAsO_4, 110 mM hydroxylamine, 1.8 mM $MgCl_2$, 0.1 mM dithiotreitol, and enzymatic extract corresponding to 100 μg protein. The incubation was performed at 37°C for 30 min. The reaction was blocked by adding 0.75 mL of 0.6 mM $Fe(NO_3)_3$ dissolved in 40% (w/v) trichloroacetic acid. The incubation mixture was centrifuged at 800*g* for 10 min and then the supernatant optical density was determined at 510 nm by referring the obtained results to a calibration curve prepared with known concentrations of L-γ-glutamil-hydroxamate. The GS activity is expressed as μmol of formed L-γ-glutamil-hydroxamate/30 min/mg protein.[26]

Astrocyte Permeability and $^{45}Ca^{2+}$ Uptake

Untreated rat primary cortical astrocytes were incubated in DMEM. The desired amount of free or liposome-associated idebenone and 40 kBq/mL $^{45}CaCl_2$ were added to the cell suspensions and incubated at 37°C for 3 min. The final sample volume was 1 mL at a $^{45}Ca^{2+}$ concentration of 1.8 mM. Astrocytes were centrifuged (25,000*g* for 30 min at 5°C) and the supernatants were quantitatively removed. Pellets were placed in scintillation vials and their radioactivity was determined. This experiment was also performed in the presence of unloaded pegylated liposomes to evaluate the effect of formulation on astrocyte membrane permeability.

Cellular Delivery of Idebenone

The cellular bioavailability of idebenone-loaded liposomes or the free drug was evaluated on primary cortical astrocytes cultured in DMEM with 10% (v/v) fetal calf serum. Cells were split once weekly. Cells were detached by using trypsin, seeded in six-well plates at $\sim 10^6$ cells per well, and incubated for 24 h before use. Cell cultures were washed with DMEM, and a suitable amount of free or liposome-associated idebenone was added to obtain a final drug concentration of 5 μM, which allows an accurate cellular determination of idebenone as a function of time. After various incubation periods, the cells were washed three times with warm 20 mM HEPES buffer (pH 7.4) and lysed with chilled water (2 mL for each well). The cells were then frozen, thawed, and sonicated. The amount of idebenone accumulated within the cells was determined by high-performance liquid chromatography.[21]

Statistical Analysis

Statistical analysis of the various experimental results was performed by using one-way analysis of variance (ANOVA). *A posteriori* Bonferroni *t* test was performed to check the ANOVA test. A p value <0.05 was considered statistically significant. All the values are reported as the average ±SD.

RESULTS AND DISCUSSION

Liposome Physicochemical Characterization

The extrusion through polycarbonate filters was performed to obtain a liposome suspension with improved colloidal properties and narrow size distribution. The final liposome suspension was made up of unilamellar vesicles with a diameter of 57.3 ± 2.9 nm and a polydispersity index value of 0.02. Although the physicochemical properties of this drug can allow a high liposome entrapment,[15] the liposome formulation used throughout this study was characterized by an idebenone-lipid molar fraction of 0.05, because at molar fractions higher than 0.06 a phase segregation of the liposome phospholipid matrix was observed.[15] Idebenone-loaded liposomes were suspended in PBS or in the presence of 20% (v/v) fetal calf serum and stored at 4°C and at room temperature for a month to evaluate the stability of the formulation. No significant variation of the liposome mean size and no idebenone leakage from liposomes were observed.

The pegylation of colloidal carriers can improve biopharmaceutics features, namely, tissue adhesion and delivery.[27] An effective pegylation of the liposome colloidal suspension should ensure a highly hydrophilic and uncharged surface. For this reason, zeta potential experiments were performed. As shown in Figure 1, the liposome colloidal suspension without MPEG-2000-DSPE has a zeta potential value of −41.7 mV. When MPEG-2000-DSPE is added to the liposome lipid composition, a reduction of the absolute value of the zeta potential is observed as a function of the pegylated phospholipid concentration, up to a molar ratio of 0.7. Above this value, a plateau (−3.7 mV) was observed, because of the complete

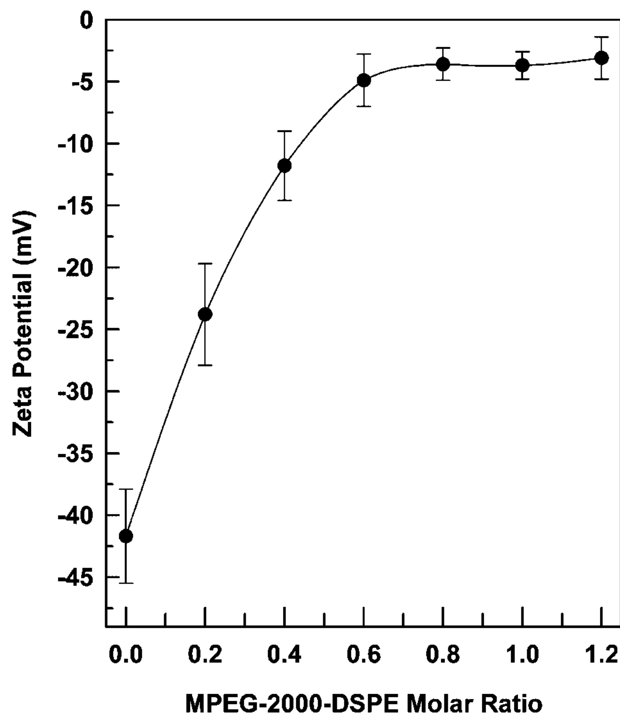

Figure 1. Zeta potential values of unilamellar liposome colloidal suspensions (~50 nm) prepared as a function of the MPEG-2000-DSPE molar ratio. The experiments were performed in distilled water.

coating of the liposome surface by the polyethylene glycol (PEG) moieties. Thus, in this study, liposomes were prepared with a MPEG-2000-DSPE molar ratio of 1 (10% mol).

Idebenone Effects on Astrocytes

Cortical astrocyte cultures were treated with both free and pegylated liposomally entrapped idebenone to evaluate the presence of possible toxic effects due to the drug, the liposome carrier, or both. Astrocytes were treated with three different drug concentrations, that is, 0.5, 5, and 50 μM. The astrocyte health and viability after the various treatments were determined by evaluating the respiratory capacity (MTT assay) and the percentage of LDH released into the culture medium.[28] GS levels were determined to evaluate the effect of environmental injury stimuli on astrocyte metabolic activity.[22] HSP70 expression was evaluated as a marker of cellular reactivity against oxidative stress.[28]

As reported in Table 1, free idebenone showed a concentration-dependent toxic effect on cortical astrocytes. The drug added to astrocytes at a concentration of 0.5 or 5 μM showed no toxic effect compared with the control, whereas 50 μM idebenone induced an increase of LDH release, a signal of toxic effects on cellular membranes. The highest concentration of idebenone did not seem to influence astrocyte viability considering that no MTT reduction was observed with respect to the control.

The pegylated liposome formulation at the highest lipid concentration used in our *in vitro* experiments (0.75 mg/mL; this is the lipid amount to obtain a 50 μM idebenone-loaded liposome

Table 1. Levels of LDH Release and MTT Assay of Primary Cortical Rat Astrocyte Cultures Treated with Free or Liposomally Entrapped Idebenone at Various Concentrations

Sample	LDH[a]	MTT[b]
Control[c]	10.1 ± 1.5	0.831 ± 0.022
Idebenone (0.5 μM)	10.7 ± 1.3	0.851 ± 0.021^{d}
Idebenone (5 μM)	9.2 ± 2.1	0.998 ± 0.015^{e}
Idebenone (50 μM)	$18.8 \pm 1.6^{e,f}$	$0.874 \pm 0.018^{d,e}$
Unloaded liposomes (0.75 mg/mL)	$15.7 \pm 0.9^{e,f}$	0.773 ± 0.020^{e}
Idebenone–liposome (0.5 μM)	11.8 ± 1.4^{g}	0.847 ± 0.017^{d}
Idebenone–liposome (5 μM)	12.1 ± 1.8^{g}	0.863 ± 0.018^{d}
Idebenone–liposome (50 μM)	$28.8 \pm 1.5^{e,h}$	$0.515 \pm 0.034^{e,h}$

Each value is the average of five different experiments ±SD. Statistical analysis was performed by ANOVA.
[a]LDH values are expressed as percentage released into the culture medium.
[b]MTT values are expressed as absorbance units at 570 nm.
[c]Untreated astrocyte cultures.
[d]$p \leq 0.001$ with respect to 5 μM free drug and unloaded liposomes.
[e]$p < 0.005$ with respect to the control.
[f]$p < 0.001$ with respect to 0.5 μM and 5 μM free drug.
[g]$p < 0.005$ with respect to 50 μM free drug and unloaded liposomes.
[h]$p < 0.001$ with respect to all other samples.

suspension) affected the astrocyte viability and caused an increase of LDH release (Table 1), probably due to a lipid exchange phenomenon occurring between pegylated liposome phospholipid bilayers and astrocytes. Pegylated liposomes at a lipid concentration $\leq$0.6 mg/mL elicited no significant effect on both LDH release and MTT levels with respect to control (data not shown). To evaluate whether this effect depends on lipid composition of liposome suspensions, liposomes only made up of DPPC were tested and a similar effect on primary astrocyte cell cultures was observed (data not reported).

Idebenone–liposome formulations at 0.5 and 5 μM concentrations induced no significant variation of both MTT values and LDH release compared with the control. A reduced cell viability (reduced MTT) was observed in the case of the 50 μM idebenone–liposome formulation (Table 1). In this case, the higher LDH release with respect to that observed for 50 μM free drug was probably due to two different factors: i) the effect of phospholipids of the pegylated liposome formulation on astrocyte membranes, and ii) the improved delivery and the increased incorporation of the drug within membrane structures caused by the pegylated liposome formulation, as demonstrated by astrocyte drug accumulation experiments (see Cellular Delivery of Idebenone).

The other two parameters investigated in astrocytes were GS activity and HSP70 levels (Figs. 2 and 3). Idebenone (50 μM), either free or liposomally entrapped, exerted a toxic effect on astrocytes by increasing HSP70 levels and significantly reducing GS activity. Conversely, low doses of idebenone, particularly when entrapped in pegylated liposomes, moderately stimulated the astrocytes' defensive capacities and improved the resistance of the cells versus different stress types (Fig. 3).

Cellular functionality was also evaluated by using morphological analysis of primary cortical astrocyte cultures. As shown in Figure 4b, astrocytes treated with 5 μM idebenone showed no significant difference with respect to untreated astrocytes (control, Fig. 4a). Treatment with 50 μM idebenone caused a substantial change of the astrocytes' morphology (Fig. 4c). The presence of 5 μM idebenone-loaded pegylated liposomes in the culture medium did not induce any modification in the normal morphology of cortical astrocytes (Fig. 4d). The highest concentration (50 μM) of the idebenone-pegylated liposome formulation induced wrinkling of the astrocytes that then

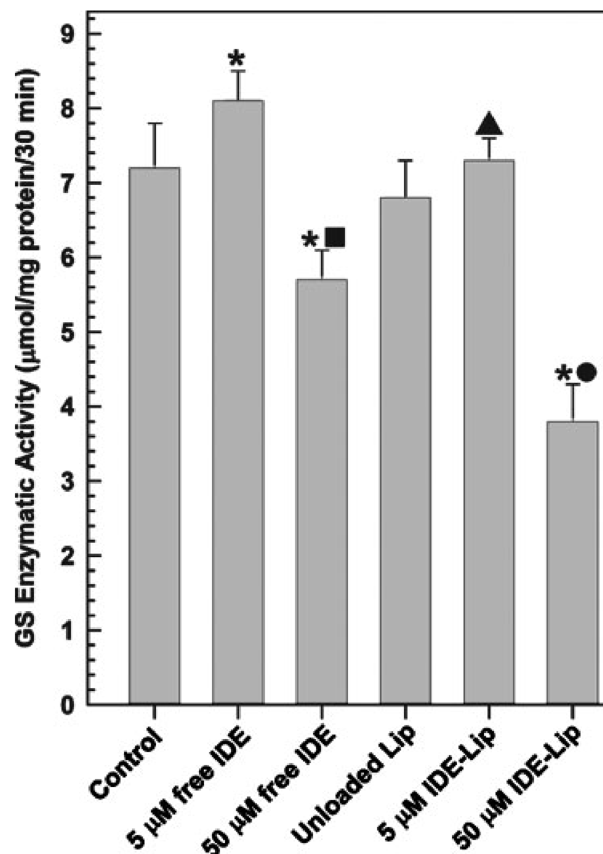

Figure 2. GS enzymatic activity in rat astrocyte cultures as a function of various treatments. Control is untreated astrocytes. Unloaded pegylated liposome (Lip) formulation has a lipid concentration of 0.75 mg/mL. Each value is the average of five different experiments ±SD. Statistical analysis by ANOVA: *, $p < 0.05$ with respect to the control; ■, $p < 0.001$ with respect to the treatment of 5 μM free idebenone (IDE); ▲, $p < 0.01$ with respect to the free drug treatments; ●, $p < 0.001$ with respect to all other treatments. Idebenone at 0.5 μM (both free and entrapped) shows no significant effect on astrocyte cells (data not reported).

began to detach from the plate (Fig. 4e). The simple liposome formulation caused no morphological change with respect to the control; this was also true for 0.5 μM free or pegylated liposome-entrapped drug (data not reported). A 50-times-higher concentration of unloaded pegylated liposomes elicited damage to the astrocytes, which appeared partially detached and had an ameboidal or macrophagical cell appearance (Fig. 4f).

Idebenone Effect on Permeability of Astrocytes

Further evidence of the effect of free or liposomally entrapped idebenone on biological mem-

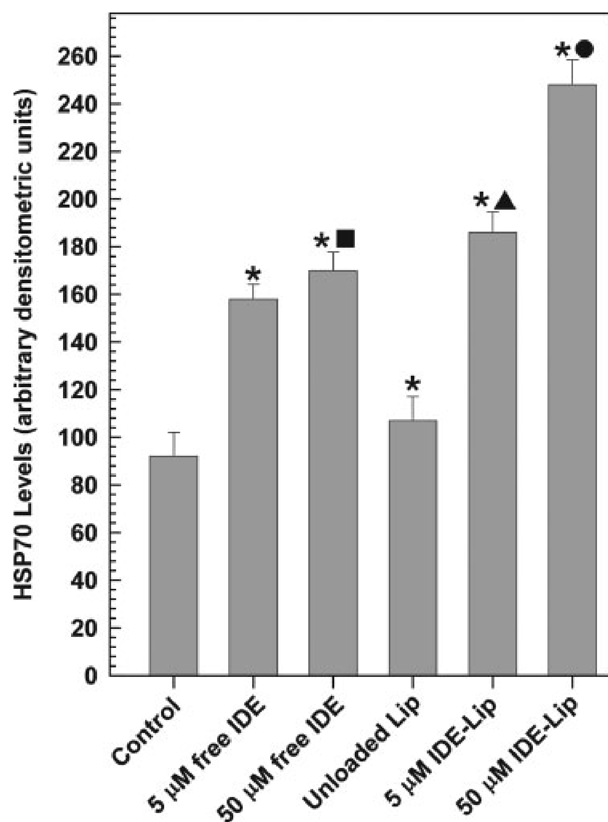

Figure 3. Densitometric analysis of constitutive and inducible HSP70 expression in rat astrocyte cultures as a function of various treatments. Control is untreated astrocytes. Unloaded pegylated liposome (Lip) formulation has a lipid concentration of (0.75 mg/mL). Each value is the average of five different experiments ±SD. Statistical analysis by ANOVA: *, $p < 0.05$ with respect to the control; ■, $p < 0.05$ with respect to the treatment of 5 μM free idebenone (IDE); ▲, $p < 0.02$ with respect to the free drug and unloaded liposome treatments; ●, $p < 0.001$ with respect to all other treatments. Treatment with idebenone at 0.5 μM (both free and entrapped) provided similar levels of HSP70 to the group treated with 5 μM free drug (data not reported).

branes was achieved by evaluating the influence on the permeability of $^{45}Ca^{2+}$ through cellular membranes of astrocytes.

As shown in Figure 5, an increased Ca^{2+} permeability was observed in astrocytes. The increase of membrane permeability, elicited by the drug, is a concentration-dependent event. The $^{45}Ca^{2+}$ uptake of cortical astrocytes showed a half-maximum effect at an idebenone concentration of 12 μM. Also in this experiment, the idebenone–liposome formulation elicited a greater permeability increase than the free drug with a half-maximum effect at 9 μM.

The idebenone-mediated increase of ion fluxes through the plasma membrane of astrocytes can be attributed to the activation/inhibition of ion transport systems and/or to a membrane perturbation effect. The sigmoidal shape of $^{45}Ca^{2+}$ uptake by astrocytes demonstrated a rapid increase of permeability beyond a certain idebenone concentration. These data are in agreement with our previous observations regarding the membrane-perturbation effect of idebenone.[15] Considering that the unloaded liposomes have no significant effect on astrocyte permeability, the difference in $^{45}Ca^{2+}$ uptake curves between the free and liposomally entrapped idebenone-loaded pegylated liposomes is due to the greater amount of the drug delivered into the membranes by the liposomal carrier.

These findings can explain the toxic effect of idebenone at a high concentration on astrocytes, namely, the perturbed membrane permeability can lead to a loss of cellular homeostasis because of decreased cellular functionality.[29]

Cellular Delivery of Idebenone

To evaluate the influence of the liposomal carrier on the delivery of the drug, cortical astrocytes were incubated with the free drug or idebenone-loaded pegylated liposomes. As demonstrated by idebenone accumulation experiments (Fig. 6), idebenone-loaded pegylated liposomes provided the highest cellular levels of the drug into the bilayer structures of astrocyte membranes with a more rapid delivery rate than the simple aqueous suspension of the free drug.

The physical mixture of the free drug and the unloaded pegylated liposome suspension showed no significant idebenone accumulation profile with respect to the free drug (Fig. 6). Therefore, the improved transport of idebenone within cells is due to the liposomal colloidal carrier, which can improve the drug diffusion through aqueous environments and to the drug interaction with cellular membranes by means of polyoxyethylene glycol moieties present on the liposome surface.[30]

Effect of Free and Encapsulated Idebenone on Ethanol-Stressed Astrocytes

The biological effectiveness of both free and liposomally entrapped idebenone at concentrations ranging from 0.05 to 50 μM was evaluated with regard to ethanol-induced cellular damage. As

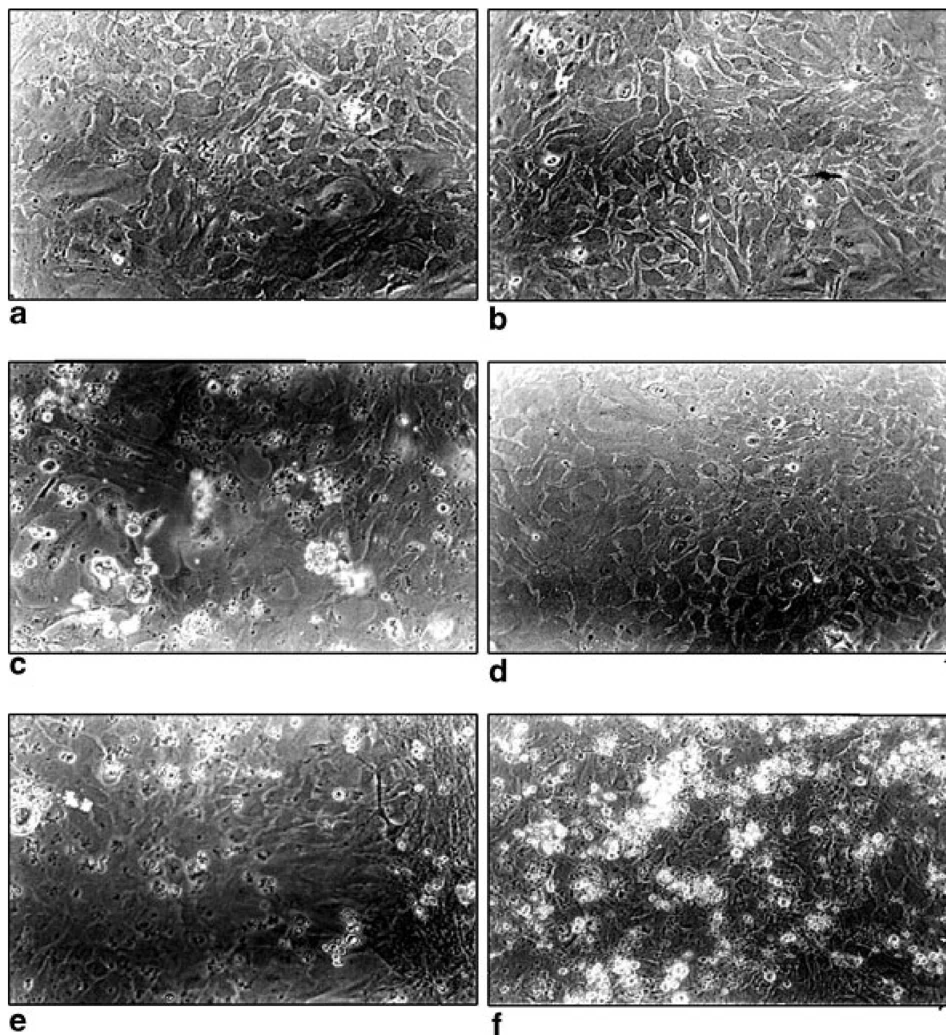

Figure 4. Optical microscopy of various primary cortical astrocyte cultures. Untreated astrocytes, control (a); astrocytes treated with 5 μM (b) or 50 μM (c) free idebenone; astrocytes treated with 5 μM (d) or 50 μM (e) idebenone-loaded pegylated liposomes; liposome formulation at a concentration 50 times higher than that used in the experiments (f).

shown in Figure 7, ethanol caused an increase of LDH release and a reduction of cell viability (MTT assay).[14,31] In our experiments, the ethanol treatment also determined an increase of HSP70 levels and a significant reduction of cellular functionality (Figs. 7 and 8).

The addition of free idebenone provided a concentration-dependent effect on ethanol-damaged astrocytes. As concerns LDH release (Fig. 7), no effect was observed at concentrations up to 0.5–1 μM. In the concentration range 1–10 μM, a progressive decrease of LDH release was observed. At concentrations higher than 10 μM, a reversal of the drug effect was observed, i.e., the LDH release began to increase. Therefore, free idebenone showed the best effect against the excitotoxic damage caused to astrocytes by chronic ethanol exposure, at a concentration of 10 μM. Similar dose-effect profiles were observed with regard to cellular viability (MTT assay) and activity (GS assay). In both cases, the best biological effect of the free drug to increase cellular viability and to improve cellular activity was observed at a concentration of 10 μM. No significant difference was observed in the level of HSP70 with respect to ethanol-injured astrocytes (Fig. 8) up to a concentration of 1 μM. When ethanol-injured primary cortical astrocytes were treated with free

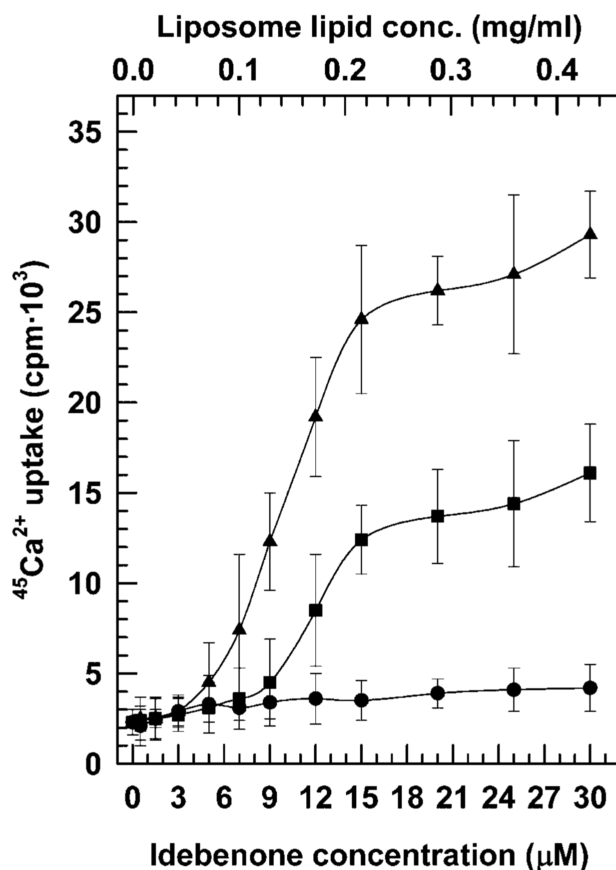

Figure 5. Uptake of $^{45}Ca^{2+}$ by primary cortical astrocytes as a function of the free drug (■), idebenone-loaded liposomes (▲), and unloaded liposomes (●). The incubation time was 5 h at 37°C. Data represent the mean value ±SD of three different experiments.

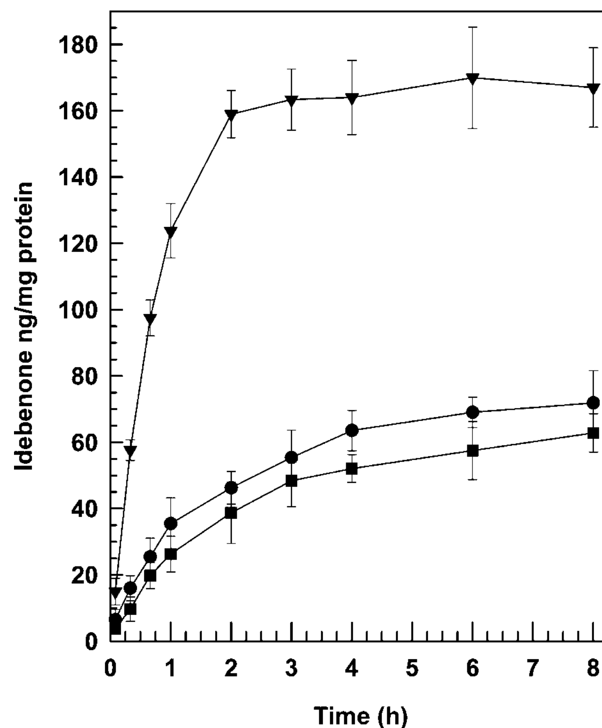

Figure 6. Amount of drug in primary cortical rat astrocytes as a function of time. Cells were incubated at 37°C with free idebenone (●), idebenone-loaded pegylated liposomes (▼), and idebenone/liposome physical mixture (■). In the various experiments, the final idebenone concentration was 5 μM. Each value is the average of five experiments ±SD. Statistical analysis by ANOVA showed $p < 0.001$ for idebenone-loaded liposomes with respect to other two curves.

idebenone at concentrations higher than 1 μM, an increase of the HSP70 levels was observed (Fig. 8).

The treatment of ethanol-injured astrocytes with unloaded liposomes caused a reduction of LDH release (Fig. 7) and an increase of HSP70 levels (Fig. 8). MTT assay (Fig. 7) demonstrated that the unloaded liposome formulation improved the viability of ethanol-damaged cells. No significant effect was observed regarding GS activity (Fig. 7). Therefore, a small positive biological effect seems to be exerted by the unloaded liposome formulation.

Idebenone-loaded pegylated liposomes were much more effective in antagonizing the cellular damage determined in astrocytes by chronic exposure to ethanol than the free drug. The best biological response of ethanol-injured primary cortical astrocytes after idebenone-loaded pegylated liposome treatment was in terms of both dose-effect profile and absolute therapeutic effect (Figs. 7 and 8).

Analyzing the astrocyte cellular parameters investigated in this study, it can be noted that the greatest therapeutic effect was obtained at an idebenone-loaded pegylated liposome concentration of 1 μM, i.e., a drug concentration 10 times lower than free idebenone. Furthermore, the therapeutic effect is greater than that observed when ethanol-injured astrocytes are treated with free idebenone, namely, the treatment with idebenone-loaded liposomes (1 μM) was able to return the LDH, MTT, and GS values of damaged astrocytes to the levels of the control (Fig. 7).

As shown in Figure 8, the increase of HSP70 levels in ethanol-injured astrocytes treated with idebenone-loaded pegylated liposomes (at concentrations higher than 0.1 μM) was significantly ($p < 0.001$) smaller than that observed for the treatment with the free drug. The level of HSP70

showed that idebenone-loaded pegylated liposomes (1 μM) are able to increase the cellular reactivity against oxidative stress. In fact, a slightly higher level of HSP70, a family of specific stress-induced proteins, with respect to the control is representative of an active and protective cellular reaction/response to different environmental and chemical insults.[32,33]

Also, in the case of ethanol-injured astrocyte treatment with idebenone-loaded pegylated liposomes, a drug concentration-dependent effect was

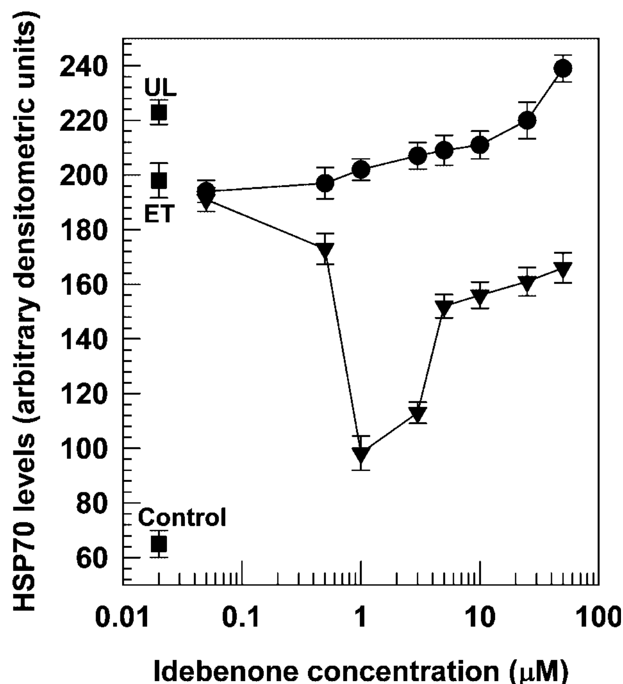

Figure 8. Effect of free or liposomally entrapped idebenone on HSP70 levels of ethanol-injured primary cortical astrocytes of rat as a function of the drug concentration. The control value comes from untreated astrocytes. The ET value shows the HSP70 level after the chronic exposure (12 days) of primary cortical astrocytes to ethanol (100 μM). UL values show the effect of unloaded pegylated liposomes on ethanol-injured astrocytes. Symbols: ●, free drug; ▼, idebenone-loaded pegylated liposomes. Each value is the average of nine different experiments ±SD.

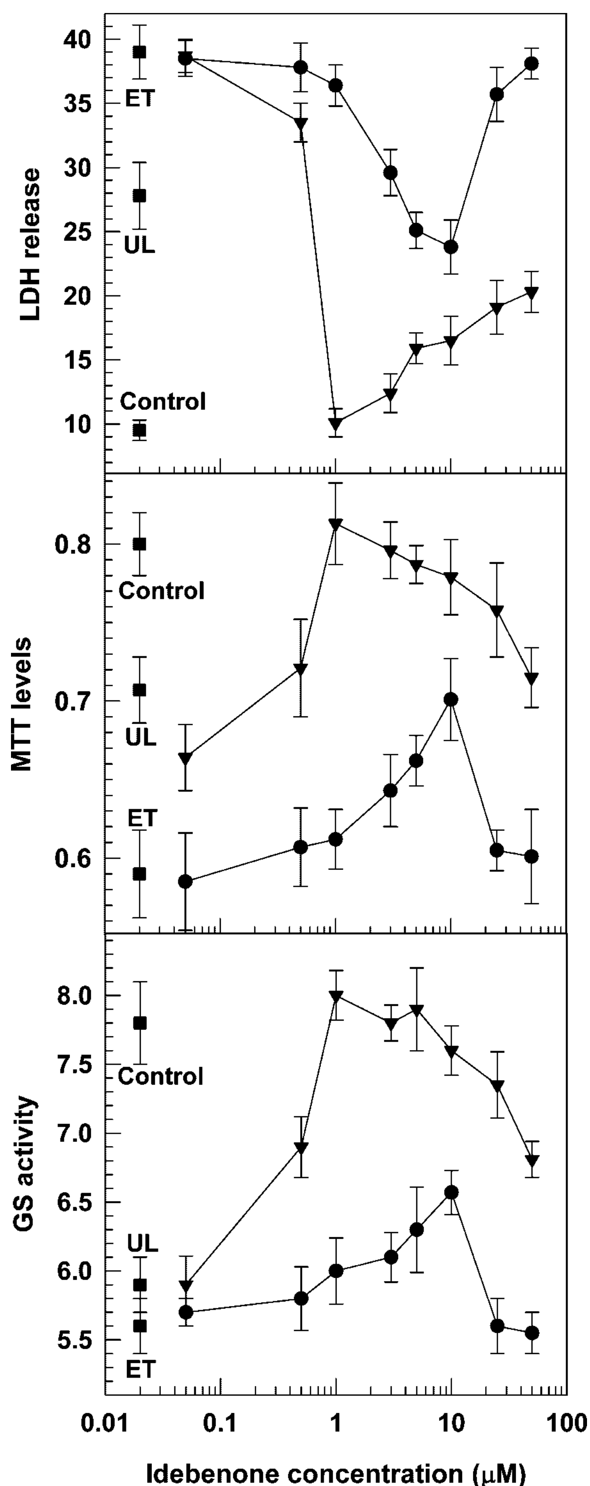

Figure 7. Biological effectiveness of free or liposomally entrapped idebenone on LDH release, MTT assay, and GS activity ( ) of ethanol-injured primary cortical astrocytes of rat as a function of the drug concentration. The control value comes from untreated astrocytes. The ET values show the results following the chronic exposure (12 days) of primary cortical astrocytes to ethanol (100 μM). UL values show the effect of unloaded pegylated liposomes on ethanol-injured astrocytes. LDH values are expressed as percentage released into the culture medium. MTT values are expressed as absorbance units at 570 nm. GS activity is expressed as $\mu mol \cdot mg\ protein^{-1} \cdot 30\ min^{-1}$. Symbols: ●, free drug; ▼, idebenone-loaded pegylated liposomes. Each value is the average of nine different experiments ±SD.

observed, i.e., at concentrations higher than 1 μM, a worsening of the therapeutic response was observed (Figs. 7 and 8). It is noteworthy that the worst therapeutic response observed for the treatment with idebenone-loaded pegylated liposomes (50 μM) was in any case significantly (ANOVA $p < 0.001$) better than that observed for the best free idebenone treatment (10 μM).

Results reported herein show that idebenone seems to have two distinct effects on biological membranes, both related to its physicochemical properties: i) a perturbation effect on the cellular membrane, and ii) an antioxidant activity on cellular and subcellular membranes due to the ubiquinone structure. The perturbation effect can explain the potentially toxic effect of the drug,[29] whereas the idebenone antioxidant activity allows the counteraction of the production of ROS, which are important mediators in the damage caused by ethanol.[34] This action is responsible for the pharmacological effects of this drug. This particular scenario can explain the concentration-dependent effects of idebenone, namely, at low concentrations, the antioxidant action (therapeutic effect) prevails over the membrane perturbation phenomenon, whereas at high drug concentrations the effect on cellular membranes becomes stronger, thus leading to an impairment of cellular functionality. The occurrence of these two actions can justify the antioxidant buffering effect with respect to ethanol-induced injury in astrocytes, which occur at high drug concentrations.

CONCLUSIONS AND PERSPECTIVES

The results reported in this article show that liposomally entrapped idebenone efficaciously antagonizes the ethanol-induced injury in primary cortical astrocytes. The improved action of idebenone when entrapped in pegylated liposomes is probably due to the greater drug bioavailability at the cellular level. The greater idebenone-loaded pegylated liposome biological effectiveness, in terms of both biological response and effective therapeutic dose, with respect to the free drug can allow the preparation of a liposomal formulation with a low drug dosage, thus improving the therapeutic index of idebenone by both the increased antioxidant activity and the reduced drug-induced toxicity at the level of cellular membranes. These encouraging results indicate the *in vivo* use of an idebenone-loaded pegylated liposome formulation in the treatment of CNS diseases mediated by the overproduction of ROS and hence by peroxidative damage.

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