*Free Radical Research*, November 2009; 43(11): 1133–1145

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# Ferulic acid inhibits oxidative stress and cell death induced by Ab oligomers: Improved delivery by solid lipid nanoparticles

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*(Received 20 May 2009; revised 24 July 2009)*

**Abstract**

Oxidative stress and dysfunctional mitochondria are among the earliest events in AD, triggering neurodegeneration. The use of natural antioxidants could be a neuroprotective strategy for blocking cell death. Here, the antioxidant action of ferulic acid (FA) on different paths leading to degeneration of recombinant $\beta$-amyloid peptide (rA$\beta$42) treated cells was investigated. Further, to improve its delivery, a novel drug delivery system (DDS) was used. Solid lipid nanoparticles (SLNs), empty or containing ferulic acid (FA-SNL), were developed as DDS. The resulting particles had small colloidal size and highly negative surface charge in water. Using neuroblastoma cells and rA$\beta$42 oligomers, it was demonstrated that free and SLNs-loaded FA recover cell viability. FA treatment, in particular if loaded into SLNs, decreased ROS generation, restored mitochondrial membrane potential ($\Delta\psi_m$) and reduced cytochrome *c* release and intrinsic pathway apoptosis activation. Further, FA modulated the expression of Peroxiredoxin, an anti-oxidative protein, and attenuated phosphorylation of ERK1/2 activated by A$\beta$ oligomers.

**Keywords:** *Ferulic acid, solid lipid nanoparticles, Alzheimer's disease, beta-amyloid, oligomers*

**Abbreviations:** *A$\beta$, amyloid-beta peptide; AD, Alzheimer's disease; BBB, blood–brain barrier; CNS, central nervous system; DCF, 2′,7′-dichlorofluorescein; DDS, drug delivery systems; FA, ferulic acid; MPS, mononuclear phagocyte system; NDGA, nordihydroguaiaretic acid; PCS, photon correlation spectroscopy; PDI, polydispersity index; ROS, reactive oxygen species; rA$\beta$42, recombinant amyloid-beta peptide 42; SLNs, solid lipid nanoparticles*

## Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by the deposition of extracellular amyloid-beta peptide (A$\beta$) and intracellular neurofibrillar tangles, associated with loss of neurons in the brain and consequent learning and memory deficit [1]. A$\beta$ peptide is the major component of the senile plaques and is believed to play a central role in the development and progress of AD both in oligomer and fibril forms. Although the aggregation of $\beta$-amyloid is thought to be a critical step in the pathogenesis of AD, recently it has been suggested that the precursors of fibrils, oligomers of A$\beta$, might be more toxic than fibrils [2–4].

Inhibition of the formation of A$\beta$ fibrils as well as the destabilization of preformed A$\beta$ in the CNS would be an attractive therapeutic target for the treatment of AD. Moreover, a large number of studies indicate that oxidative stress may play an important role in the aetiology and progression of AD due to the destructive effect of the mitochondrial integrity and the perturbation of cellular energy homeostasis [5].

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ISSN 1071-5762 print/ISSN 1029-2470 online 
DOI: 10.1080/10715760903214454

Peroxides like $H_2O_2$ are produced as the result of normal cellular processes involving oxygen. The long-term presence of even a small amount of those peroxides is a risk to cells because they can be converted into toxic radicals and thereby damage cellular components. Besides the peroxides produced at basal levels, cells produce $H_2O_2$ transiently in response to the activation of various stimuli. Cells are therefore equipped with peroxide-eliminating enzymes like catalase, glutathione peroxidase and peroxiredoxins [6]. Increased production of reactive oxygen and nitrogen species coinciding with a depletion of antioxidant defences is observed in neuronal systems after A$\beta$ treatment [7].

Free radicals are chemical species with unpaired electrons or highly reactive molecules that cause oxidative stress. This is defined as 'an imbalance between oxidants and antioxidants in favour of the oxidants, potentially leading to damage' (Sies 1997, page 291) [8]. Oxidative stress can damage lipids, proteins, enzymes, carbohydrates and DNA in cells and tissues, resulting in membrane damage, fragmentation or random cross-linking of molecules such as DNA, enzymes and structural proteins. Oxidative stress may even lead to cell death induced by DNA fragmentation and lipid peroxidation [9]. Moreover, it is known that ROS are able to elicit apoptosis in a large variety of cultured cells of different origins.

A promising preventive or therapeutic intervention for AD patients could be the suppression or the reduction of the oxidative stress. Many antioxidant compounds, indeed, such as vitamin E [10], nordihydroguaiaretic acid (NDGA) and nicotine have been demonstrated to protect the brain from A$\beta$ neurotoxicity [11,12]. Ferulic acid (FA), (4-hydroxy-3-methoxycinnamic acid), is an antioxidant naturally present in plant cell walls. It has a phenolic nucleus and a long side chain so it readily forms a resonance stabilized phenoxy radical with high antioxidant [13,14] and anti-inflammatory activities [15,16]. It has been suggested that FA can act as a free radical scavenger [13,17–19]. Further, administration of FA induces resistance to A$\beta$42 toxicity in mice and it has been suggested that it may be a useful chemopreventive agent against AD [20]. Moreover, inhibition of the formation as well as destabilization of preformed amyloid fibrils has been suggested [21].

A central problem in the treatment of brain disorders is to reach a suitable drug amount into the brain, due to the presence of the blood–brain barrier (BBB). Several DDS have been developed to circumvent the BBB, such as niosomes, liposomes, polymeric micelles, polymeric and lipid nanoparticles [22]. In recent years the attention has focused on SLN which are particles with a solid lipid matrix and an average diameter in the nanometer range. SLN are made from highly purified triglycerides, complex glyceride mixtures or even waxes. These systems have many advantages such as: the possibility of controlled drug release and drug targeting, increased drug stability, high drug loading capacity, the feasibility of incorporating lipophilic and hydrophilic drugs, the biocompatibility of the carrier and no problems with respect to large-scale production and sterilization [22–24]. Because of their small size, these systems may be injected intravenously, avoiding the uptake of macrophages of mononuclear phagocyte system (MPS). Furthermore, their lipophilic features could lead them to CNS crossing BBB by means of the endocytotic mechanism of the endothelial cells lining the brain blood capillaries [22–27].

A$\beta$42 forms, *in vitro*, fibrils that are similar to those found in Alzheimer's plaques. Depending on pH value, it is possible to obtain samples containing mainly small oligomers or fibrils [28,29]. In our previous work, using a recombinant A$\beta$42 peptide (rA$\beta$42) that presents biophysical and immunological properties comparable to those of the natural peptide [30], we have demonstrated that, under physiological pH conditions, rA$\beta$42 forms small oligomers, whereas larger aggregates are formed at acidic pH. Moreover, we have recently demonstrated that on LAN5 neuroblastoma cells rA$\beta$42 fibrillar aggregates stimulate extrinsic apoptotic pathway via caspase 8 activation, whereas rA$\beta$42 oligomers principally trigger intrinsic apoptotic pathway via caspase 9 activation [31].

In the present study we investigate the protective effect of FA on the oxidative stress and consequent cell death induced by rA$\beta$42 oligomers. Moreover, we verify the possibility to utilize solid lipid nanoparticles to improve the FA delivery and to enhance its potential antioxidant therapeutic effect.

## Materials and methods

### *Materials*

Compritol 888 ATO (mixture of approximately mono-, di- and tri-glycerides of behenic acid at 15, 50 and 35% w/w), was obtained from Gattefossè Italia s.r.l. (Milan, Italy). Epikuron 200 (soybean phosphatidylcholine, minimum 95wt% phosphatidylcholine) was a kind gift from Lucas Meyer (Hamburg, Germany); taurocholate sodium salt was a kind gift from Prodotti Chimici e Alimentari S.p.A. (Basaluzzo-Alessandria, Italy). FA was purchased from Fluka (Milan, Italy). The other chemicals, of analytical grade, were obtained from Sigma (Milan Italy).

### *Preparation of empty and FA-loaded SLNs*

Empty and drug-loaded SLNs were prepared via a warm oil-in-water (o/w) microemulsion by using Compritol 888 ATO as lipid matrix, Epikuron 200 as a surfactant, taurocholate sodium salt as a

cosurfactant. The lipid phase was prepared by heating Compritol 888 ATO (0.246 mmol) ~5–10°C above its melting point. Successively, an aqueous suspension (0.65 μl) of Epikuron 200 (0.049 mmol) and an aqueous solution (0.34 ml) of taurocholate sodium salt (0.243 mmol) were added, thus obtaining a clear microemulsion. SLNs were obtained by dispersing the warm o/w microemulsion in cold water (100 g) at 3 ± 1°C, under mechanical stirring. FA-loaded SLNs were obtained by dissolving FA in the melted lipid matrix and operating as described above. The obtained aqueous dispersions of SLNs were centrifuged, at 4°C, at 45 000 rpm for 1 h, suspended in water and recovered after freeze-drying by a Modulyo freeze-dryer (Edwards, Crawley, UK) for successive characterization.

*Particle size determination*

The mean diameter and width of distribution (polydispersity index, PDI) of empty and FA-loaded SLNs were determined by photon correlation spectroscopy (PCS). The measurements were carried out by using a Zetasizer Nano ZS (Malvern Instrument, Herrenberg, Germany) which utilizes the Non-Invasive Back-Scattering (NIBS) technique. Each sample was appropriately diluted with filtered (0.2 μm) double-distilled water. The measurements were done at a scattering angle of 173°. Each suspension was put in a cuvette and analysed in triplicate.

*Zeta potential measurements*

The zeta potential was measured using principles of laser Doppler velocitometry and phase analysis light scattering (M3-PALS technique). For this purpose, a Zetasizer Nano ZS Malvern Instrument equipped with a He-Ne laser at a power P = 4.0 mW and with $\lambda = 633$ nm was used. Empty and FA- loaded SLNs were dispersed in filtered (0.2 μm) double-distilled water or an aqueous solution of NaCl (0.9% w/v) or phosphate saline buffer (PBS) at pH 7.4, with conductivity adjusted to 50 μS/cm. Each sample was analysed in triplicate.

*Determination of loading capacity (LC%)*

To determine the amount of FA entrapped into SLNs, 5 mg of freeze-dried SLNs were dissolved in 25 ml of tetrahydrofuran (THF). The organic solution was filtered through 0.45 μm (PTFE membrane) filters and analysed by HPLC. In order to ensure that the drug is not absorbed on the PTFE membrane, several THF solutions of drug at known concentrations were filtered and the concentration values before and after filtration were evaluated by HPLC analysis. No significant differences in drug concentration was observed. Results were expressed as LC% (w/w), that is the relative percentage of loaded drug with respect to the lipid phase (matrix lipid + drug).

The HPLC analysis was performed at room temperature using a Shimazu Instrument equipped with a reversed-phase $C_{18}$ column (Bondpak, 3 m, 150 × 4.6 mm i.d., Supelco). A mixture of tetrahydrofuran (THF), $H_2O$ and $CH_3COOH$ (60:35:5 v/v) with a flow rate of 0.2 ml/min was used as mobile phase. The HPLC column system was connected with a UV-Vis detector (Shimazu). The drug peak was measured at a wavelength of 318 nm and quantitatively determined by comparison with a standard curve obtained using FA solutions in THF at known concentrations.

*Drug release at pH 7.4*

FA release from SLNs was assessed *in vitro* by a dissolution method, using United States Pharmacopoeia (USP) apparatus II. A dissolver (Esadissolver 3) was fitted with six 1 L dissolution vessels and apparatus II paddle set. The paddle rotation speed was set at 50 rpm and the vessels, containing the dissolution medium PBS 0.01 M at pH 7.4, were kept at 37°C. At selected time intervals, 50 mg of formulation were added to each vessel and samples of 3 ml volume were withdrawn, filtered through 0.22 μm (nylon membrane) and analysed by HPLC.

*Cell cultures*

LAN5 human neuroblastoma cell line was plated into 96-well plates at a density of $1.5 \times 10^4$ per well for 24 h. Cells were cultured with RPMI 1640 medium (CELBIO) supplemented with 10% foetal bovine serum (FBS) (GIBCO) and 1% antibiotics (50 μg/ml penicillin and 50 μg/ml streptomycin) and antimycotics (SIGMA). Cells were maintained in a humidified 5% $CO_2$ atmosphere at 37 ± 0.1°C. Empty SLNs and different amounts (14 μM, 28 μM) of FA, free or loaded into SLNs (dispersion volumes: 5 μl or 10 μl), were incubated together with 10 μM rA$\beta$42 on LAN5 cells for 24 h. The recombinant A$\beta$42 peptide (rA$\beta$42) was produced, purified and prepared under oligomeric form according to Carrotta et al. [29]. The treated cultured cells and the controls were morphologically analysed by microscopy inspection, using *a Leica* inverted microscope, or utilized for specific assays. For the ROS, JC-1, Caspase activation assays and Western blot experiments we utilized FA at 28 μM, free or loaded into SLNs (dispersion volume: 10 μl).

*Determination of cell viability*

Cell viability was measured by MTS assay (PROMEGA). MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphopheyl)2H-tetrazolium] was utilized according to the manufacturer's instructions. After cells treatments, 20 μl of the MTS

solution were added to each well and the incubation was continued for 4 h at 37°C, 5% $CO_2$. The absorbance was read at 490 nm on the Microplate reader $^{wallac}$Victor $^{2}$ 1420 MULTILABEL COUNTER (Perkin Elmer). Results were expressed as the percentage MTS reduction of the control cells.

*Analysis of mitochondrial transmembrane potential generation and reactive oxygen species (ROS)*

Mitochondrial membrane potential was measured directly using the MitoProbe JC-1 Assay Kit (Molecular Probes, Eugene, OR). LAN5 human neuroblastoma cell line were untreated (Control) or treated with 10 μM rA$\beta$42 oligomers alone or combined with free FA (28 μM) or with FA (28 μM) blended into SLNs (dispersion volume: 10 μl) for 24 h. Afterwards, the cells were incubated with 2 μM JC-1 (5,5′,6,6′-tetrachloro-1,1′;,3,3′-tetraethylbenzimidazolylcarbocyanine iodide) fluorescent dye in PBS (137 mM NaCl, 2.7 mM KCl, 8 mM $Na_3PO_4$, pH 7.4) for 30 min at 4°C. CCCP (carbonyl cyanide 3-chlorophenylhydrazone) (50 μM), a mitochondrial membrane potential disrupter, was used as control and to perform standard compensation (data not shown). Fluorescence emission shift of JC-1 from red (~ 590 nm) to green (~ 529 nm) was evaluated by flow cytoflourimetry (FACScalibur, Becton Dickinson, Mountain View, CA) with 488 nm excitation laser.

To assess ROS generation by flow cytofluorimetry, cells were incubated for 24 h as mentioned above. Afterwards, cells were incubated with 1 μM dichlorofluorescein diacetate (DCFH-DA) in PBS for 10 min at room temperature in the dark.

After washing, cells were dissolved in PBS and then analysed by flow cytometry for fluorescence-positive cells analysis. Gate on the cells, excluding debris, was performed from forward (FS) and sideways (SS) scatter patterns.

*Caspase assays*

Caspase-9, 3/7 activities in cells were measured using commercially available luminescent assays (caspase–Glo$^{TM}$ 9, caspase–Glo$^{TM}$ 3/7 assay systems, PROMEGA). LAN5 cells were untreated (control) or treated with 10 μM oligomers alone or with FA at 28 μM, free or loaded into SLNs (dispersion volume: 10 μl) for 4 h. Caspase reagent specific for each kit was added directly to the cells in white 96-well plates and, after mixing, they were incubated for 15–30 min before recording luminescence with $^{wallac}$Victor$^{2}$ 1420 MULTILABEL COUNTER (Perkin Elmer) apparatus. The caspase activator assay was performed utilizing 50 μM vinblastine (Sigma, Milan, Italy). The caspase inhibitor assay was performed with 50 μM Z-VAD (Promega).

*Cytochrome c release assay.* LAN5 cells were untreated (control) or treated with 10 μM oligomers alone or with FA at 28 μM, free or loaded into SLNs (dispersion volume: 10 μl) and separately were collected. Cytosol and mitochondria fractions were prepared utilizing a Mitochondria Isolation Kit (PIERCE) and according to the manufacturer's instructions. Lysates of both the fractions and controls were separated on 4–20% SDS PAGE (Cambrex) and the Western blot was incubated with 1:1000 diluted anti-cytochrome *c* (Cell Signalling Technology).

*Protein extraction and Western blotting*

Total proteins were prepared by dissolving in lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5% Triton X-100, 2 mM PMSF, 10 μg/ml protease inhibitor, 1 mM $Na_3VO_4$, 1 mM NaF) LAN5 cells untreated (control) or treated with 10 μM oligomers, alone or with FA at 28 μM, free or loaded into SLNs. Cells to be used as control were treated with $H_2O_2$ 2 mM for 20 min., as free radical generator. Protein samples (50 μg) were electrophoretically separated using 10% SDS-PAGE gel and transferred onto nitrocellulose filters for immunoblotting. After blocking in 3% BSA in TBST, the Western blot was incubated with anti-phosphorylated ERK 1/2 (1:1000; SIGMA) or anti-peroxiredoxine 3 (1:1000; Sigma, Milan, Italy). Primary antibodies were detected using the ECL chemiluminescence kit (Amersham) according to the manufacturer's instructions and using secondary antibodies conjugated to horseradish peroxidase (1:1500) (Amersham). Bands intensities were analysed with a gel documentation system (BioRad). Prx3 and ERK1/2 expressions were adjusted to actin expression. The protein levels were expressed as densitometry and percentage of controls.

*Statistical analysis*

All experiments were repeated at least three times. Each experiment was performed in triplicate. The results are presented as mean ± SD. Statistical evaluation was conducted by ANOVA, followed by Student's *t*-test for analysis of significance. Results with a $p$-value $< 0.05$ were considered statistically significant.

## Results

*Production and characterization of SLNs*

Empty and FA-loaded SLNs potentially to be used as DDS were prepared successfully via a warm o/w microemulsion by using Compritol 888 ATO. The obtained nanoparticles were characterized in terms of particle size, PDI, zeta potential, LC% and drug release profile. In Table I, mean diameter and PDI values are reported. The average diameter of either

Table I. Mean size, PDI and LC% of empty and FA-loaded SLNs. Each value is the mean of three experiments ± SD.

| | Size (NM) | PDI | LC%(w/w) |
|---|---|---|---|
| Empty SLNs | 85.36 ± 1.85 | 0.256 ± 0.031 | / |
| FA-loaded SLNs | 96.16 ± 2.96 | 0.196 ± 0.038 | 20.0% |

empty or drug-loaded SLNs was quite small, 85.36 and 96.16 nm, respectively, the empty system being smaller than that drug-loaded. The quite small PDI values indicate a good dimensional homogeneity of both systems. Moreover, the LC% of FA-loaded SLNs, determined by HPLC analysis and expressed as the percentage of loaded drug with respect to the lipid phase (matrix lipid + drug), resulted very high (20.0% w/w).

To investigate the surface charge of empty and FA-loaded SLNs, zeta potential measurements were carried out both in double-distilled water and in isotonic media with physiological fluids (NaCl 0.9%, PBS at pH 7.4). Table II shows the zeta potential values of empty and FA-loaded SLNs. The surface charge values are negative for both empty and drug-loaded samples in all the investigated media. Moreover, these values are larger in double-distilled water for both empty and drug-loaded SLNs, while they are smaller in NaCl 0.9% and PBS at pH 7.4 because of the shielding effects due to the presence of ions in solutions (Table II).

In addition, the evaluation of FA release was performed in simulated extracellular fluid (PBS at pH 7.4). The release profile, reported in Figure 1, shows a complete drug delivery within 10 h. Although a relevant burst release was observed, ~ 40 wt% of drug incorporated into the lipid matrix was released in a prolonged way.

All the physicochemical characteristics of these SLNs suggest that they can be used for a potential intravenous administration and, due to their small size, the nanoparticles could probably minimize the attack of macrophages of MPS and reach the CNS, so overcoming the BBB.

### *FA, free or loaded into SLNs, protects cell against rAβ42 oligomer toxicity*

To test the activity of free FA against A$\beta$ toxicity and investigate if the entrapment of FA into SLNs may improve the effectiveness of free FA, we carried out *in vitro* experiments on LAN5 neuroblastoma cells

Table II. Zeta potential values (mV) of empty and FA-loaded SLNs. Each value is the mean of three experiments ± SD.

| | $H_2O$ | NaCl 0.9% | PBS (pH 7.4) |
|---|---|---|---|
| Empty SLNs | −28.24 ± 3.15 | −3.56 ± 0.18 | −8.45 ± 0.35 |
| FA-loaded SLNs | −36.40 ± 1.38 | −1.97 ± 0.25 | −11.7 ± 2.24 |

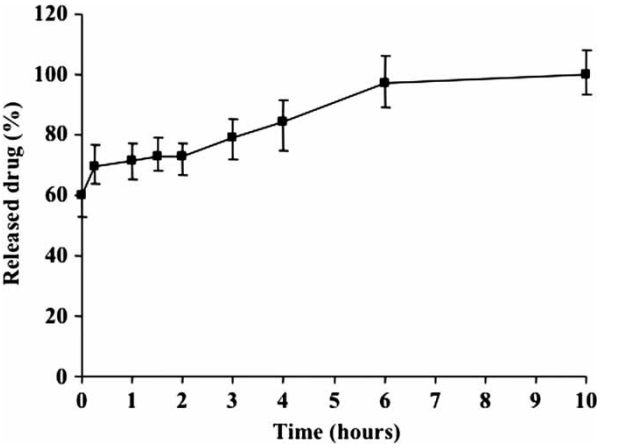


Figure 1. Release profiles of FA from FA-loaded SLNs in PBS at pH 7.4. Each value is the mean of six experiments.

treated with rA$\beta$42 oligomers. As a first approach, the morphological effect with respect to the corresponding controls (Figure 2A–C) was observed. Representative images of neurons treated with rA$\beta$42 oligomers at various stages of degeneration are shown in Figure 2D. Morphological changes resulted in a reduction of the cellular body, neuritis and neuronal cell number. Cells treated with rA$\beta$42 oligomers and FA free (2E) or loaded into SLNs (2F), instead, appear to recover the regular morphology of neurons.

To confirm the morphological results and to evaluate the amount of FA, free or entrapped into SLNs, a dose-response study was performed. To this purpose various amounts of FA, in both conditions, were incubated with rA$\beta$42 treated LAN5 cells for 24 h. All the treated cells and the control were examined by MTS assay.

As shown in Figure 3, cells treated with rA$\beta$42 alone or with empty SNLs showed a mortality of ~ 40% if compared with the control. When free FA, at concentrations of 14 μM and 28 μM, was utilized a recovery of cell viability of ~15% and 20%, respectively, was observed. Further, when FA at the highest concentration (28 μM) entrapped into SLNs was employed, a best result, in terms of cell viability, was obtained. In fact, a total recovery of rA$\beta$42-induced toxicity was detected, indicating that these SLNs are good carriers for improving the protective effect of FA.

### *FA-loaded SLNs restore mitochondrial membrane potential and inhibit Aβ42-induced intracellular ROS generation*

At physiological membrane potential JC-1 forms red-fluorescent aggregates. Therefore, intact living cells stained with JC-1 exhibit a red fluorescence, detectable by flow cytometry analysis. On the other hand, stressed cells result in a break-down of the mitochondrial membrane potential and a prevalent increase of green fluorescence. Consequently, mitochondrial

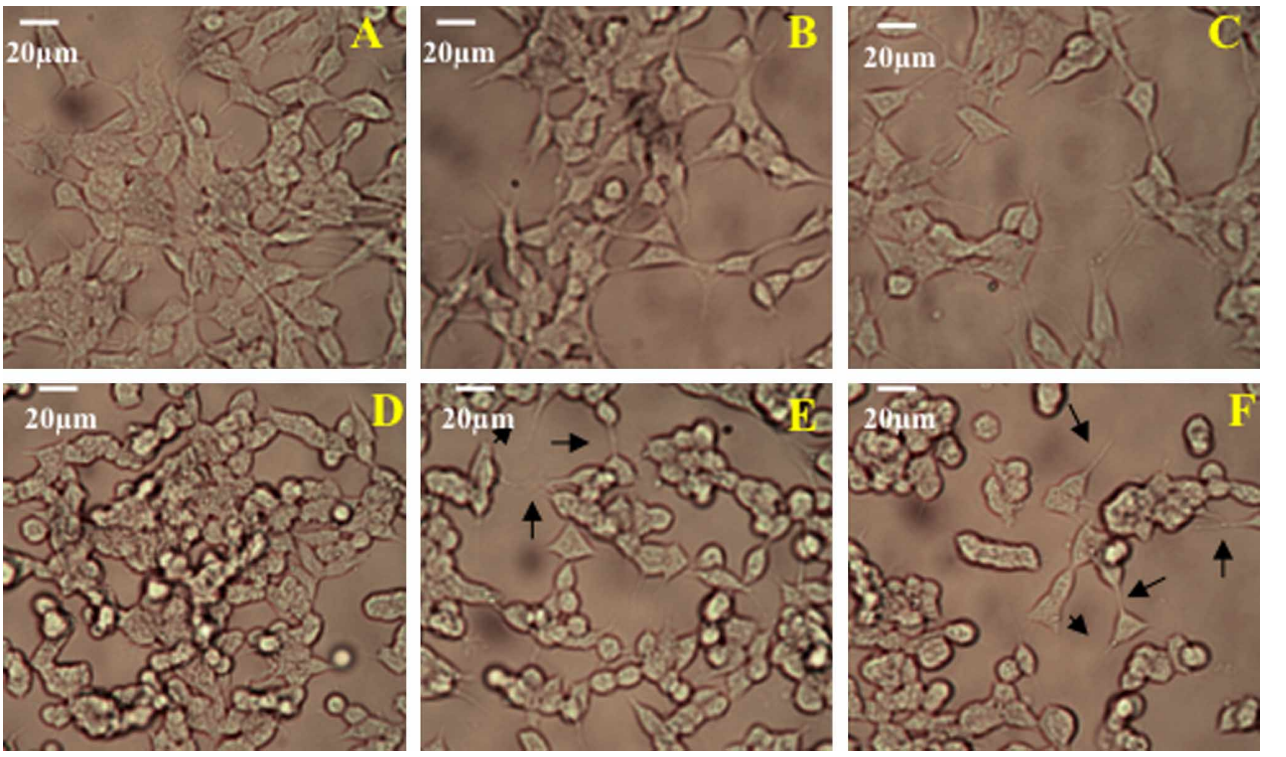


Figure 2. rA$\beta$42 oligomers produce morphological changes to LAN5 neuroblastoma cells. Images of LAN5 cells untreated (A), treated with free FA (B), treated with FA-loaded SLNs (C), treated with rA$\beta$42 oligomers (D), incubated with rA$\beta$42 oligomers and free FA (E) or FA-loaded SLNs (F). The arrows indicate the neurites. Bar: 20 µm.

depolarization is indicated by a decrease in the red/green fluorescence intensity ratio.

The mitochondrial membrane potential ($\Delta\Psi_m$) of LAN5 cells treated with rA$\beta$42 alone or together with FA, free or entrapped into SLNs, was monitored by measuring the fluorescence of JC-1 monomers. The mitochondrial membrane was found to be significantly depolarized by rA$\beta$42 treatment (Figure 4A). Moreover, the mitochondrial membrane potential upon rA$\beta$42 treatment in the presence of FA free or entrapped into SLNs was similar to that observed in control samples, thus indicating that FA, in both supplied conditions, restored the mitochondrial membrane potential by counteracting rA$\beta$42 activity. Further, a total recovery was observed for FA entrapped into SLNs.

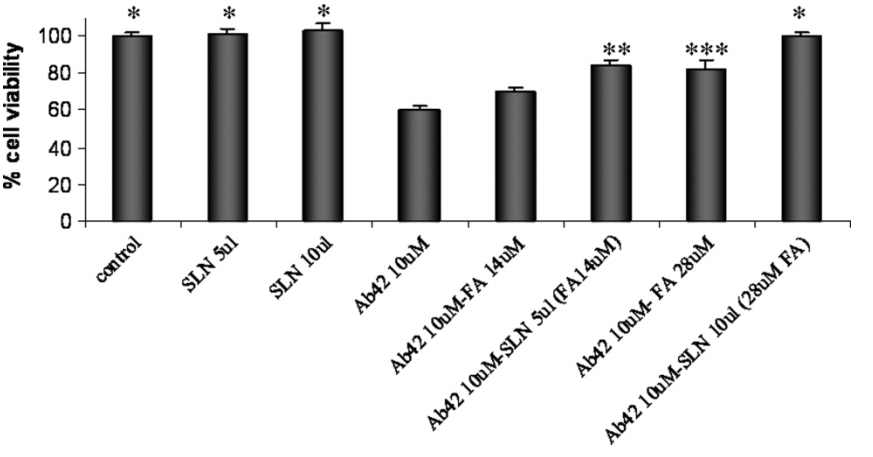


Figure 3. Dose dependence of protective effect of free Ferulic acid or FA-loaded SLNs against rA$\beta$42 oligomers induced cell death. LAN5 neuroblastoma cells were untreated (control) or incubated with 5 µl or 10 µl of empty SLNs (SLN 5 µl, SLN 10 µl) or with 10 µM of rA$\beta$42 oligomers alone (Ab42 10 µM) or with empty SLN (Ab42 10 µM SLN) or together with different doses (14 µM and 28 µM) of free FA (Ab42; FA 14 µM, Ab42; FA 28 µM) or FA loaded with 5 µl of SLN (Ab42 10 µM; SLN 5 µl; FA 14 µM) or 10 µl of SLN (Ab42 10 µM; SLN 10 µl; FA 28 µM). After 24 h of incubation treated and untreated cells were submitted to viability MTS assay. Cell viability is significantly higher in control (*$p < 0.01$), in SLN 5 µl (*$p < 0.01$), in SLN 10 µl (*$p < 0.01$), in Ab42 10 µM; SLN 5 µL (14 µM FA) (** $p < 0.02$), in Ab42 10 µM; FA 28 µM (*** $p < 0.05$), in Ab42 10 µM; SLN 10 µL (28 µM FA (*$p < 0.01$) vs Ab42. Percentage of viability is referred to as control, the data are the mean ± SD of three separate experiments.

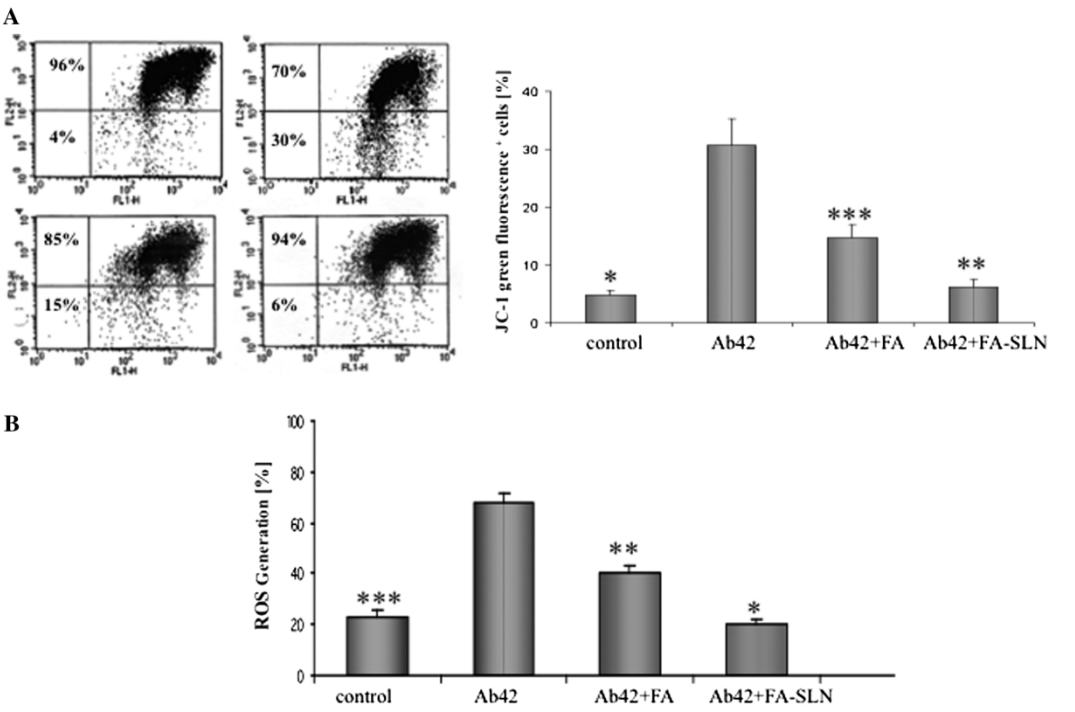


Figure 4. LAN5 neuroblastoma cells were untreated or treated with rA$\beta$42 alone or combined with free FA or FA-loaded SLNs. (A) Protection of FA free or loaded into SLNs against A$\beta$42 induced mitochondrial membrane potential change. The number indicates the percentage of green fluorescence positive cells. Right, representative graph of flow cytometric analysis. Mitochondrial membrane is significantly depolarized by rA$\beta$42 treatment vs control, $^{*}p < 0.002$, vs free FA, $^{***}p < 0.01$, and vs FA-loaded SLNs, $^{**}p < 0.003$. (B) The percentage of ROS generation is significantly higher by rA$\beta$42 treatment vs control, $^{***}p < 0.05$, free FA, $^{**}p < 0.02$, and FA-loaded SLNs, $^{*}p < 0.01$.



Furthermore, some evidences suggest that A$\beta$ is able to generate free radical and oxidative damage [5]. Then, we have also investigated the ability of FA, free or loaded into SLNs, in reducing ROS produced by A$\beta$ treatment. Intracellular ROS were measured by dichlorofluorescein diacetate (DCFH-DA) cytofluorometric assay. The conversion of non-fluorescent dichlorofluorescein diacetate (DCFH-DA) to the highly fluorescent compound 2′,7′-dichlorofluorescein (DCF) by cellular esterase activity can be used to monitor the presence of peroxides due to the oxidative burst in LAN5 cell line. Therefore the emitted fluorescence is directly proportional to the concentration of hydrogen peroxide inside the cell. Here we investigate if FA, free or entrapped into SLNs, is able to inhibit rA$\beta$42-induced intracellular ROS formation. rA$\beta$42 oligomers induced a significantly higher ROS percentage generation with respect to the other three conditions (Figure 4B). In particular in the presence of FA entrapped into SLNs, rA$\beta$42-induced intracellular ROS formation was completely inhibited. A ROS percentage generation similar to that found under control conditions was observed, so confirming the antioxidant proprieties of FA. The weak activation of ROS observed in the control is probably due to basal metabolism.

### *FA-loaded SLNs reduce rAβ42 induced release of cytocrome c*

As a consequence of mitochondrial membrane depolarization, cytochrome *c* could be released from the intermembrane space of mitochondria in the cytosol. In order to compare the effects on mitochondrial membrane of the treatment with rA$\beta$42 oligomers alone, or with rA$\beta$42 in the presence of FA or FA-loaded SLNs, a cellular fractionation was performed. Mitochondria and cytosol fractions were obtained by LAN5 cells untreated or incubated with oligomers alone, oligomers with FA and with FA-loaded SLNs. Proteins from all these samples were extracted and the presence of cytochrome *c* in each fraction was analysed by Western blot, using a specific antibody. Figure 5 shows that in untreated control cells, cytochrome *c* and actin are exclusively present in the mitochondrial or cytosolic fractions, respectively. Instead, in oligomers treated cells a decrease in the mitochondrial pool of cytochrome *c* was detected and in contrast a significant amount of cytochrome *c* was present in the cytosol. Furthermore, in LAN5 cells treated with rA$\beta$42 oligomers and FA a weak band in cytosol proteins was detected, whereas no band was detected in cytosol proteins when LAN5 cells were treated with rA$\beta$42 oligomers and FA-loaded SLNs. This result

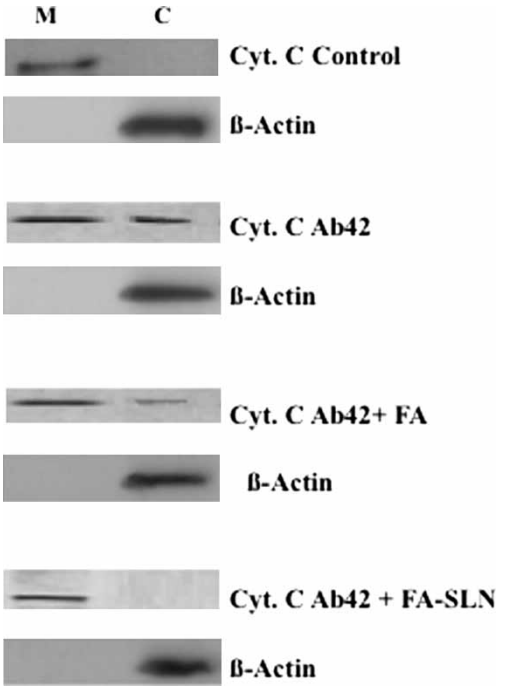


Figure 5. Free FA or loaded into SLNs reduce cytochrome *c* release produced by oligomers in LAN5 cells. Western blot of proteins extracted from mitochondrial (M) and cytosolic (C) fractions of LAN5 untreated cells (control) or treated with oligomers alone (Ab42) and together free FA (Ab42+FA) or FA-loaded SLNs (Ab42+FA-SLN) and incubated with anti-cytochrome *c* (cyt. C) or anti-*β*-actin (*β*-actin) as control.

suggests that these two treatments interfere with the rA*β*42 oligomers action, producing a protection of the mitochondrial membrane.

### *FA-loaded SLNs reduce apoptosis induced by Aβ42 oligomers*

Proteolytic enzymes of the caspase family play a central role in initiating and sustaining the event leading to apoptotic cell death. In particular, intrinsic pathway, activated by caspase 9, involves the mitochondria. To investigate about the possibility that FA, free or incorporated into the SNLs, can interfere with apoptotic process activated by rA*β*42, we utilized a Caspase 9 luminometric assay on LAN5 cells treated with rA*β*42 oligomers alone or in the presence of FA or FA-loaded SLNs (Figure 6A). A caspase activator was employed as a control. As can be seen in Figure 6, cells treated with oligomers in the presence of FA or FA-loaded SLN showed a strong reduction of activation of caspase 9 with respect to cells treated with oligomers alone, indicating that the drug induced a positive protective effect. In order to confirm this result, LAN5 cells treated as described above were put through to caspase 3 assay, the typical caspase executrix. As expected, in the LAN5 cells treated with oligomers and FA or FA-loaded SLNs a drastic reduction in the caspase 3 activation respect to the rA*β*42 cells was detected (Figure 6B). Moreover, these results confirm that A*β* oligomers induce intrinsic apoptotic pathway and the compound here presented can down-regulate caspase activation.

### *FA modulates Peroxiredoxin 3 expression*

Cells possess an anti-oxidation defence system. Peroxiredoxin 3 (Prx3) is a protein having peroxide reductase activity [32]. It is considered the most important $H_2O_2$-eliminating enzyme in mitochondria. Increased Prx3 expression and the ability to maintain Prx3 in a reduced state seem to be a neuroprotective mechanism occurring in response to A*β* toxicity [33]. To determine whether FA, free or entrapped into SLNs, interferes in Prx3 expression, a Western blot experiment was performed. Proteins extracted by LAN5 cells treated with rA*β*42 oligomers with or without FA free and FA-loaded SLNs and with $H_2O_2$, as control, were immobilized into a membrane and incubated with anti-Prx3. An intense double band of 23 and 26 kDa, corresponding to the two isoforms of Prx3 [33], was detected in $H_2O_2$ and rA*β*42 treated cells (Figure 7A). A double band with a lower intensity, not comparable to that of the untreated cells, was instead detected in the samples treated with FA and FA-loaded SLNs. Increased Prx3 expression suggested that cellular ROS defence mechanism was activated but it was not sufficient to balance the ROS over-production induced by rA*β*42. Cells treated with rA*β*42, indeed, as shown before (Figure 6), activate cell death pathway. In contrast, the decreased Prx3 level in samples treated with rA*β*42 and FA free or SLN loaded suggested that the reduced mitochondrial ROS generation due the presence of free FA or FA-loaded SLNs required a lower Prx3 production.

### *FA-loaded SLNs reduce ERK activation induced by Aβ42 oligomers*

Among the mitogen-activated kinase (MAPK) family, a role for the extracellular signal-regulated kinase (ERK)1/2 pathway, in AD neuronal death, has been proposed [34]. Activation of ERK seems in turn activates caspase-3 [35]. Moreover, ERK activation is also associated with cell death induced by ROS [36]. FA being able to inhibit ROS generation and reverse caspases 9 and 3 activation, we investigated about the possibility to obtain some effect on ERK 1/2-signalling. A Western blot of proteins extracted after A*β* treatment with or without FA free or loaded into SLNs was incubated with antibodies against activated ERK1/2. Consistent with other studies, LAN5 treated with A*β* resulted in the sustained activation of ERK1/2 as compared with the untreated control (Figure 8). As expected, FA free and loaded into SLNs consistently reduced A*β* mediated activation of ERK1/2.

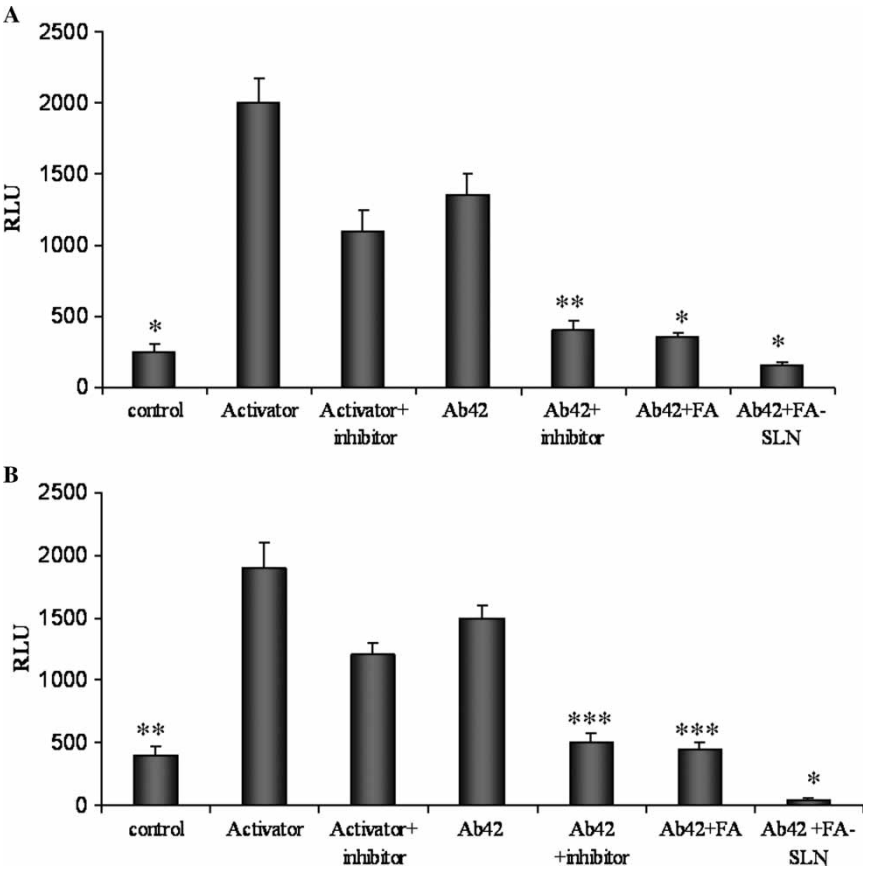


Figure 6. FA and FA-loaded SLNs inhibit apoptosis induced by rA$\beta$42 oligomers. LAN5 cells were untreated or treated with rA$\beta$42 oligomers alone (Ab42) or with free FA (Ab42-FA) or FA-loaded SLNs (Ab42SLN-FA) or with vinblastine (activator) or with Z-VAD-FMK (inhibitor) and submitted to caspase 9 (A) or 3/7 (B) luminescent assays. Caspase reagent specific for each kit was added directly to the cells in white 96-well plates and after mixing were incubate for 15–30 min, before recording luminescence with appropriate apparatus. Inhibition of caspase 9 (A) and 3/7 (B) activation, induced by rA$\beta$42 was observed after incubation of LAN5 cells with oligomers and FA and FA-loaded SLNs. On the left the RLU (Relative Light Units) are indicated. Caspase9 (A) activation is significantly lower in control ($*p < 0.01$), in Ab42+FA ($*p < 0.01$), in Ab42+FA-SLN ($*p < 0.01$) and in Ab42+inhibitor ($**p < 0.02$) vs rAb42. Caspase3 (B) activation is significantly lower in control ($**p < 0.02$), in Ab42+FA ($***p < 0.05$), in Ab42+FA-SLN ($*p < 0.01$) and in Ab42+inhibitor ($***p < 0.05$) vs rAb42.

## Discussion

The intraneuronal accumulation of A$\beta$, implicated in AD, is generated in physiological or pathological conditions within intracellular sites as endoplasmic reticulum, Golgi apparatus, endosomal-lysosomal systems [37]. Moreover, some evidences indicate that monomers and oligomers of A$\beta$ are associated to mitochondria in transgenic mouse model and AD brain [37–42]. Here, utilizing rA$\beta$42 oligomers and neuroblastoma cells, we mimic the *in vivo* condition, in order to investigate about the connection of some pathways, involving mitochondria and leading to neurodegeneration. We also investigated if these pathways can be reversed by using an antioxidant such as FA. Moreover, we demonstrate that an enhanced FA action can be obtained when FA-loaded SLNs are employed, and we provide some evidence that FA-loaded SLNs can be delivered and utilized at mitochondrial level.

The need to develop and improve new strategies for the release of therapeutic and diagnostic agents has been widely recognized. In this effort we have prepared by means of microemulsion techniques new nanoparticles based on solid lipids (SLNs), potentially useful as DDS for FA. The use of SLNs as DDS offers many advantages such as the possibility of controlled drug release and drug targeting, increased drug stability, high drug loading capacity and lack of biotoxicity of the carrier [24]. In particular, we used Compritol 888 ATO as lipid matrix, Epikuron 200 as surfactant and sodium taurocholate as cosurfactant. The amount of FA entrapped into drug-loaded sample (LC%), expressed as the percentage of loaded drug with respect the lipid phase (matrix lipid + drug), was 20% w/w. After preparation and purification, the obtained samples were characterized in terms of mean size, PDI and zeta potential, indicating that the average diameter of nanoparticles is found to range from 85–96 nm, with the empty system being smaller

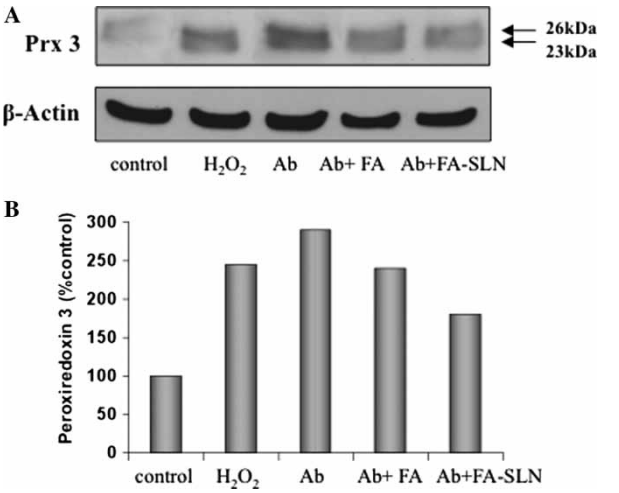


Figure 7. Prx3 expression in presence of rA$\beta$42 without/with ferulic acid. (A) Western blot of proteins extracted by untreated LAN5 cells (control) or treated with $H_2O_2$ ($H_2O_2$) or treated with rA$\beta$42 (Ab) and treated with rA$\beta$42 and FA (Ab+FA) or treated with rA$\beta$42 and FA loaded on SLNs (Ab+ FA-SLN). On the right molecular weight. Uniformity of gel loading was confirmed with $\beta$-actin as standard. (B) Quantification of immunoreactivity was performed using densitometric analysis.



than drug-loaded SLNs. This result is probably due to the larger total lipid amount precipitated in the aqueous cold phase for obtaining drug-loaded nanoparticles, as reported by other authors [43–45]. Moreover, the quite low PDI values indicate a good homogeneity degree of the system dimensions. A complete drug release from FA-loaded SLNs was obtained within 10 h. The release profile appeared suitable for using this system also *in vivo*, since once in the blood stream, the migration into the brain must be very fast. Moreover the system, due to its small size, could minimize the attack of macrophages of MPS and quickly reach the CNS, overcoming the BBB probably by an endocytosis mechanism [21–26,46,47]. Here, SLNs-entrapped FA could perform its antioxidant action against the damage produced by A$\beta$.

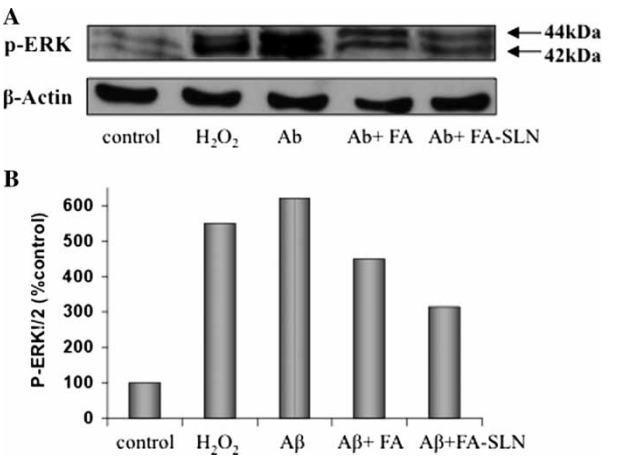


Figure 8. ERK1/2 activation in response to rA$\beta$42 oligomers without or with ferulic acid. Western blot of proteins extracted by untreated LAN5 cells (control) or treated with $H_2O_2$ ($H_2O_2$) or treated with rA$\beta$42 (Ab) or treated with rA$\beta$42 and FA (Ab+FA) or treated with rA$\beta$42 and FA loaded on SLNs (Ab+FA-SLN). On the right molecular weight. Uniformity of gel loading was confirmed with $\beta$-actin as standard. (B) Quantification of immunoreactivity was performed using densitometric analysis.

The use of antioxidants against $\beta$-amyloid induced toxicity can be a promising strategy of treatment for AD. Several *in vitro* studies have shown that synthetic A$\beta$ facilitates the generation of free radicals [48]. In contrast, some evidence has suggested that oxidative stress precedes A$\beta$ deposition and aggregate formation [49] and other authors have proposed that A$\beta$ fibrils act as free radical scavengers [50]. These results can be considered in agreement with the possibility that A$\beta$ oligomers can damage internal organelles. Although many pro-apoptotic stimuli induce the intracellular accumulation of $H_2O_2$, a direct relation between the mitochondrial generation of $H_2O_2$ and apoptosis has still not been demonstrated. An important site to be targeted in oxidative stress-related diseases is, indeed, the mitochondrion, which is the main source of ROS production. Antioxidants such as ubiquinone and vitamin E analogues, as Mito Q and Mito Vit E, were synthesized to have mitochondria as targets [51]. Here, we utilize purified rA$\beta$42 oligomers that, on the basis of the biological effects, are believed to be able to enter into cytoplasmatic environment of neuroblastoma cells. The model system used here suggested that A$\beta$, in agreement with other studies, directly interacts with mitochondria [42,48–52]. This interaction promotes the leakage of ROS, mitochondrial dysfunction, cytochrome *c* release and apoptotic cell death, events that may underline the mechanism of A$\beta$-induced mitochondrial toxicity in AD progression.

When FA is employed, in A$\beta$-treated cells, a normalized mitochondrial membrane potential ($\Delta\psi_m$), a decreased ROS generation (Figure 4), a decreased cytochrome *c* release (Figure 5) and an inhibition of caspase 9 and caspase 3 activation (Figure 6) are observed. Thus, FA inhibited oxidative stress and the cascade of events leading to cellular death. The inhibitor effect was enhanced when FA was entrapped into SLNs. This result provides the evidence that ROS can act as a signalling molecule to initiate apoptosis [53] and is in agreement with studies demonstrating that the antioxidants, acting as scavengers for ROS, suppress apoptosis. Epidemiological clinical studies have demonstrated that inhibition of oxidative stress is effective in reducing the clinical manifestation of neurodegenerative diseases [54]. Moreover, an age-dependent increase in intracellular ROS may be responsible, with others events, for a major A$\beta$ production by APP cleavage, its precursor. Thus, it seems that A$\beta$ produces ROS and ROS produces A$\beta$, and on the perspective to block this

process, it becomes important to identify a DDS that can be potentially utilized for the prevention and the treatment of oxidative stress induced by A$\beta$.

For a better efficacy, the drug should reach the site of action, and the data here reported suggest that the drug carried by the investigated DDS has an effect on mitochondrion, even if it is possible that it can reach other sites or organelles. For a long time mitochondria have been widely considered 'the motor of cell death' [55], so that the drug targeting towards these structures can be regarded as a promising therapeutic strategy. Consequently, increasing efforts have been made to either design low-molecular weight molecules able to target mitochondria or to develop drug carrier systems for the selective delivery of drugs and DNA to and into mitochondria [56]. To classify molecules displaying a high degree of mitochondrial affinity, the term 'mitochondriotropics' has been proposed [56–58]. Further, for mitochondria uptake occurring, it is necessary that whole molecules possess certain physicochemical features (e.g. lipophilic character) [59] and the nanoparticles system here employed seems to satisfy them.

Further evidence that FA shows a scavenger effect for mitochondrial ROS production is the different expression of Prx3 with respect to A$\beta$ treated cells. Peroxiredoxins 3 (Prx3) belongs to a family of highly conserved proteins which catalyse the reduction of peroxides in the presence of thioredoxin and Prx3 is considered a mitochondrion-specific $H_2O_2$ scavenging enzyme [32]. Under apoptotic stimuli Prx3 is rapidly oxidized and precedes later apoptotic events including collapse of mitochondrial membrane potential, cytochome *c* release and caspase activation [60]. Results here presented show that LAN5 treated with A$\beta$, or $H_2O_2$ as control, increased Prx3 expression suggesting that when the toxic stimuli produce excessive ROS, even if a neuroprotective plan is triggered, the cellular anti-oxidant defences are not enough and cell degenerative path is activated. When exogenous FA free or loaded into SLNs is added to A$\beta$ treated LAN5 cells, expression of Prx3 is lower with respect to that of A$\beta$ treated cells, but larger with respect to that of the untreated control. Since FA free or SLN loaded decreases ROS formation, we suppose that in this condition the scavenger role of Prx3 is less necessary and the activation of the described downstream events is reduced or inhibited.

Sub-cellular compartmentalization of reactive oxygen species (ROS) plays a critical role in cell signal transmission in response to particular stimuli. It is known that A$\beta$ induced toxicity can be partially mediated through activation of the ERK1/2 signal transduction pathway, which results in caspase-3 activation and cell death [35]. Results here obtained indicate that FA provides a substantial protection against A$\beta$ injury. Further, its antioxidant properties affect other cellular mechanisms such as biochemical pathways. In this context, we found that ERK1/2 phosphorylation was reduced by free FA and improved when it was SLN loaded with respect to A$\beta$ treated cells, suggesting that FA can interfere directly or indirectly with MAPK kinase pathway and consequently with the downstream signal transduction.

Finally, we propose a model in which when an excess of mitochondrial ROS is produced, by A$\beta$ oligomers stimulus, the natural antioxidant defences provided by Prx3 and other molecules are not sufficient to avoid loss of mitochondrial membrane potential, cytochrome *c* release, caspases activation, together with ERK phosphorylation. When, at the same toxicity conditions, FA is added, better if loaded on SLNs, ROS formation is reduced and the downstream cascade of events is inhibited (Figure 9). However, we cannot exclude that FA inhibits ROS formation in other cellular compartments.

In conclusion, we report here that $\beta$-amyloid is a key factor in free radical generation and oxidative damage activating a cascade of events leading to apoptotic cell death. The suppression or the reduction of the oxidative stress seems to be a promising therapeutic intervention for neurodegenerative diseases. The use of antioxidant molecules such as FA and the proposed DDS, appear to be potentially useful for an effective therapy for AD treatment.

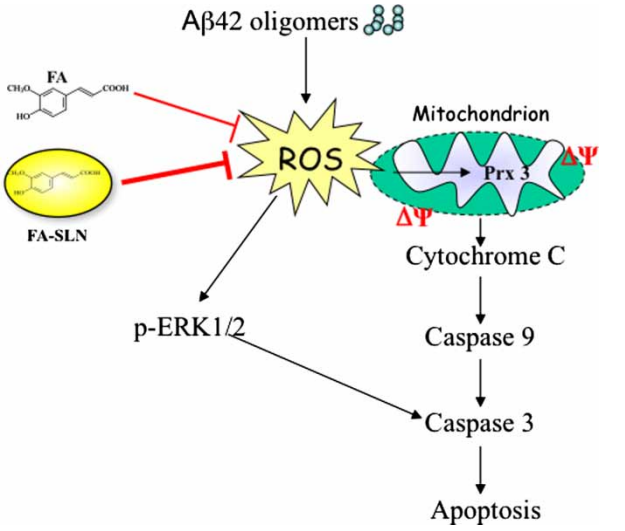


Figure 9. A scheme depiciting the protective role of Ferulic acid free or SNL loaded in response to stimulation by oligomeric A$\beta$42 in LAN5 neuroblama cells. An excess of mithochondrial ROS is produced, by A$\beta$ oligomers stimulus, the natural antioxidant defences provided by Prx3 and other molecules are not sufficient to avoid loss of mitochondrial membrane potential ($\Delta\psi$), cytochrome *c* release, caspases activation, together with ERK phosphorylation. When FA free o and particularly when loaded on SLNs is added, ROS formation is reduced and the downstream cascate of events, induced by them, inhibited.

## Acknowledgements

The authors wish to thank Dr D Giacomazza for critical reading of the manuscript.

## References

[1] Walsh DM, Selkoe DJ. A beta oligomers—a decade of discovery. J Neurochem 2007;101:1172–1184.
[2] Walsh DM, Selkoe DJ. Oligomers on the brain: the emerging role of soluble protein aggregates in neurodegeneration. Protein Pept Lett 2004;11:213–228.
[3] Pitschke M, Prior R, Haupt M, Riesner D. Detection of single amyloid beta-protein aggregates in the cerebrospinal fluid of Alzheimer's patients by fluorescence correlation spectroscopy. Nat Med 1998;4:832–834.
[4] Gong Y, Chang L, Viola KL, Lacor PN, Lambert MP, Finch CE, Krafft GA, Klein WL. Alzheimer's disease-affected brain: presence of oligomeric A beta ligands (ADDLs) suggests a molecular basis for reversible memory loss. Proc Natl Acad Sci USA 2003;100:10417–10422.
[5] Reddy PH. Amyloid precursor protein-mediated free radicals and oxidative damage: implications for the development and progression of Alzheimer's disease. J Neurochem 2006;96: 1–13.
[6] KangDagger SW, ChaeDagger HZ, SeoDagger M, KimDagger par K, Baines Dagger IC, Goo Rhee S. Mammalian peroxiredoxin isoforms can reduce hydrogen peroxide generated in response to growth factors and tumor necrosis factor-alpha. J Biol Chem 1998;273:6297–6302.
[7] Butterfield DA. Amyloid beta-peptide (1-42)-induced oxidative stress and neurotoxicity: implications for neurodegeneration in Alzheimer's disease brain. Free Radic Res 2002;361:307–313.
[8] Sies H. Oxidative stress: oxidants and antioxidants. Exp Physiol 1997;82:291–295.
[9] Beckman KB, Ames BN. The free radical theory of aging matures. Physiol Rev 1998;78:547–581.
[10] Subramaniam R, Koppal T, Green M, Yatin S, Jordan B, Drake J, Butterfield DA. The free radical antioxidant vitamin E protects cortical synaptosomal membranes from amyloid beta-peptide (25–35) toxicity but not from hydroxynonenal toxicity: relevance to the free radical hypothesis of Alzheimer's disease. Neurochem Res 1998;23:1403–1410.
[11] Goodman Y, Steiner MR, Steiner SM, Mattson MP. Nordihydroguaiaretic acid protects hippocampal neurons against amyloid beta-peptide toxicity, and attenuates free radical and calcium accumulation. Brain Res 1994;654:171–176.
[12] Kihara T, Shimohama S, Sawada H, Kimura J, Kume T, Kochiyama H, Maeda T, Akaike A. Nicotinic receptor stimulation protects neurons against beta-amyloid toxicity. Ann Neurol 1997;42:159–163.
[13] Graf E. Antioxidant potential of ferulic acid. Free Radic Biol Med 1992;13:435–448.
[14] Scott BC, Butler J, Halliwell B, Aruoma OI. Evaluation of the antioxidant actions of ferulic acid and catechins. Free Radic Res Commun 1993;19:241–253.
[15] Fernandez MA, Saenz MT, Garcia MD. Anti-inflammatory activity in rats and mice of phenolic acids isolated from Scrophularia frutescens. J Pharm Pharmacol 1998;50:1183–1186.
[16] Ozaki Y. Antiinflammatory effect of tetramethylpyrazine and ferulic acid. Chem Pharm Bull 1992;40:954–956.
[17] Kikuzaki H, Hisamoto M, Hirose K, Akiyama K, Taniguchi H. Antioxidant properties of ferulic acid and its related compounds. J Agric Food Chem 2002;50:2161–2168.
[18] Kanski J, Aksenova M, Stoyanova A, Butterfield DA. Ferulic acid antioxidant protection against hydroxyl and peroxyl radical oxidation in synaptosomal and neuronal cell culture systems in vitro: structure-activity studies. J Nutr Biochem 2002;13:273–281.
[19] Ogiwara T, Satoh K, Kadoma Y, Muratami Y, Unten S, Atsumi T, Sakagami H, Fujisawa S. Radical scavenging activity and cytotoxicity of ferulic acid. Anticancer Res 2002;22:2711–2717.
[20] Yan JJ, Cho JY, Kim HS, Kim KL, Jung JS, Huh SO, Suh HW, Kim YH, Song DK. Protection against beta-amyloid peptide toxicity *in vivo* with long-term administration of ferulic acid. Br J Pharmacol 2000;133:89–96.
[21] Ono K, Yamada M. Ferulic acid destabilizes preformed beta-amyloid fibrils *in vitro*. Biochem Biophys Res Commun 2005;336:444–449.
[22] Reddy JS, Venkateswarlu V. Novel delivery systems for drug targeting to the brain. Drugs of the Future 2004;29:63–83.
[23] Kreuter J. Nanoparticulate systems for brain delivery of drugs. Adv Drug Del Rev 2001;47:65–81.
[24] Wang J, Sun X, Zhang Z. Enhanced brain targeting by synthesis of 3′,5′-dioctanoyl-5-fluoro-2′-deoxyuridine and incorporation into solid lipid nanoparticles. Eur J Pharm Biopharm 2002;54:285–290.
[25] Wissing SA, Kayser O, Müller RH. Solid lipid nanoparticles for parenteral drug delivery. Adv Drug Del Rev 2004;56 :1257–1272.
[26] Tamai I, Tsuji A. Drug delivery through the blood–brain barrier. Adv Drug Del Rev 1996;19:401–424.
[27] Yang SC, Lu FL, Cai Y, Zhu JB, Liang BW, Yang CZ. Body distribution in mice of intravenously injected camptothecin solid lipid nanoparticles and targeting effect on brain. J Contr Rel 1999;59:299–307.
[28] Carrotta R, Manno M, Bulone D, Martorana V, San Biagio PL. Protofibril formation of amyloid beta-protein at low pH via a non-cooperative elongation mechanism. J Biol Chem 2005;280:30001.
[29] Carrotta R, Barthès J, Longo A, Martorana V, Manno M, Portale G, San Biagio PL. Large size fibrillar bundles of the Alzheimer amyloid beta-protein. Eur Byophys J 2007;36:701–709.
[30] Carrotta R, Di Carlo M, Manno M, Montana G, Picone P, Romancino D, San Biagio PL. Toxicity of recombinant beta-amyloid prefibrillar oligomers on the morphogenesis of the sea urchin Paracentrotus lividus. FASEB J 2006;20:1916–1927.
[31] Picone P, Carrotta R, Montana G, Nobile MR, San Biagio PL, Di Carlo M. Ab oligomers and fibrillar aggregates induce different apoptotic pathways in LAN5 neuroblastoma cell cultures. Biophys J 2009;96:4200–4211.
[32] Chang TS, Cho CS, Park S, Yu S, Kang SW, Rhee SG. Peroxiredoxin III, a mitochondrion-specific peroxidase regulates apoptotic signaling by mitochondria. J Biol Chem 2004;279:41975–41984.
[33] Cumming RC, Dargusch R, Fischer WH, Schubert D. Increase in expression levels and resistance to sulfhydryl oxidation of peroxiredoxin isoforms in amyloid beta-resistant nerve cells. J Biol Chem 2007;282:30523–30534.
[34] Chong YH, Shin YJ, Lee EO, Kayed R, Glabe CG, Tenner AJ. ERK1/2 activation mediates Abeta oligomer-induced neurotoxicity via caspase-3 activation and tau cleavage in rat organotypic hippocampal slice cultures. J Biol Chem 2006;281:20315–20325.
[35] Zhuang S, Schnellmann RG. A death-promoting role for extracellular signal-regulated kinase. J Pharmacol Exp Ther 2006;1:991–997.
[36] Ramachandiran S, Huang Q, Dong J, Lau SS, Monks TJ. Mitogen-activated protein kinases contribute to reactive oxygen species-induced cell death in renal proximal tubule epithelial cells. Chem Res Toxicol 2002;15:1635–1642.

[37] Hardy J, Selkoe DJ. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science 2002;297:353–356.
[38] Anandatheerthavarada HK, Biswas G, Robin MA, Avadhani NG. Mitochondrial targeting and a novel transmembrane arrest of Alzheimer's amyloid precursor protein impairs mitochondrial function in neuronal cells. J Cell Biol 2003;161:41–54.
[39] Caspersen C, Wang N, Yao J, Sosunov A, Chen X, Lustbader JW, Xu HW, Stern D, McKhann G, Yan SD. Mitochondrial Abeta: a potential focal point for neuronal metabolic dysfunction in Alzheimer's disease. FASEB J 2005;19:2040–2041.
[40] Crouch PJ, Blake R, Duce JA, Ciccotosto GD, Li QX, Barnham KJ, Curtain CC, Cherny RA, Cappai R, Dyrks T, Masters CL, Trounce IA. Copper-dependent inhibition of human cytochrome C oxidase by a dimeric conformer of amyloid-beta1-42. J Neurosci 2005;25:672–679.
[41] Wang X, Su B, Perry G, Smith MA, Zhu X. Insights into amyloid-βinduced mitochondrial dysfunction in Alzheimer disease. Free Radic Biol Med 2007;43:1569–1573.
[42] Manczak M, Anekonda TS, Henson E, Park BS, Quinn J, Reddy PH. Mitochondria are a direct site of Abeta accumulation in Alzheimer's disease neurons: implications for free radical generation and oxidative damage in disease progression. Hum Mol Genet 2006;15:1437–1449.
[43] Lim SJ, Kim CK. Formulation parameters determining the physicochemical characteristics of solid lipid nanoparticles loaded with all-trans retinoic acid. Int J Pharm 2002;243: 135–146.
[44] Ugazio E, Cavalli R, Gasco MR. Incorporation of cyclosporin A in solid lipid nanoparticles (SLN). Int J Pharm 2002;241: 341–344.
[45] Bondì ML, Craparo EF, Giammona G, Cervello M, Azzolina A, Diana P, Martorana A, Cirrincione G. Nanostructured lipid carriers-containing anticancer compounds: preparation, characterization, and cytotoxicity studies. Drug Del 2007;14:61–67.
[46] Bondì ML, Craparo EF, Giammona G, Drago F. Nanoparticulate lipid carriers containing riluzole and pharmaceutical compositions containing said particles. Patent number: WO2008/000448 A2.
[47] Beduneau A, Saulnier P, Benoit JP. Active targeting of brain tumors using nanocarriers. Biomaterials 2007;28:4947–4967.
[48] Hensley K, Carney JM, Mattson MP, Aksenova M, Harris M, Wu JF, Floyd RA, Butterfield DA. A model for beta-amyloid aggregation and neurotoxicity based on free radical generation by the peptide: relevance to Alzheimer disease. Proc Natl Acad Sci USA 1994;91:3270–3274.
[49] Praticò D, Uryu K, Leight S, Trojanoswki JQ, Lee VM. Increased lipid peroxidation precedes amyloid plaque formation in an animal model of Alzheimer amyloidosis. J Neurosci 2001;21:4183–4187.
[50] Bush AI, Atwood CS, Goldstein LE, Huang X, Rogers J. Could Abeta and AbetaPP be antioxidants? J Alzheimer Dis 2000;2:83–84.
[51] Sheu SS, Nauduri D, Anders MW. Targeting antioxidants to mitochondria: a new therapeutic direction. Biochim Biophys Acta 2006;1762:256–265.
[52] Lustbader JW, Cirilli M, Lin C, Xu HW, Takuma K, Wang N, Caspersen C, Chen X, Pollak S, Chaney M, Trinchese F, Liu S, Gunn-Moore F, Lue LF, Walker DG, Kuppusamy P, Zewier ZL, Arancio O, Stern D, Yan SS, Wu H. ABAD directly links Abeta to mitochondrial toxicity in Alzheimer's disease. Science 2004;304:448–452.
[53] Nomura K, Imai H, Koumura T, Arai M, Nakagawa Y. Mitochondrial phospholipid hydroperoxide glutathione peroxidase suppresses apoptosis mediated by a mitochondrial death pathway. J Biol Chem 1999;274:29294–29302.
[54] Münch G, Schinzel R, Loske C, Wong A, Durany N, Li JJ, Vlassara H, Smith MA, Perry G, Riederer P. Alzheimer's diseases-synergistic effects of glucose deficit. Oxidative stress and advanced glycation endproducts. J Neural Transm 1998;105:439–461.
[55] Brown GC, Nicholls DG, Cooper CE. Mitochondria and cell death. Princeton, NJ: Princeton University Press; 1999.
[56] Weissig V, Cheng SM, D'Souza G. Mitochondrial pharmaceutics. Mitochondrion 2004;3:229–244.
[57] Weissig V, Torchilin VP. Mitochondriotropic cationic vesicles: a strategy towards mitochondrial gene therapy. Curr Pharm Biotechnol 2000;1:325–346.
[58] Horobin RW, Trapp S, Weissig V. Mitochondriotropics: a review of their mode of action, and their applications for drug and DNA delivery to mammalian mitochondria. J Contr Rel 2007;121:125–136.
[59] Smith RA, Porteous CM, Gane AM, Murphy MP. Delivery of bioactive molecules to mitochondria *in vivo*. Proc Natl Acad Sci USA 2003;100:5407–5412.
[60] Cox AG, Pullar JM, Hughes G, Ledgerwood EC, Hampton MB. Oxidation of mitochondrial peroxiredoxin 3 during the initiation of receptor-mediated apoptosis. Free Radic Biol Med 2008;44:1001–1009.

This paper was first published online on iFirst on 14 September 2009.