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Abstract

Lipid microparticles (LMs) uncoated or coated with chitosan and containing the neuroprotective polyphenol, resveratrol were developed for its targeting to the brain via nasal administration. The lipid microparticles loaded with resveratrol (LMs-Res) were produced by melt emulsification, using stearic acid as lipid material and phosphatidylcholine as the surfactant. The chitosan coated particles LMs-Res-Ch (1.75% w/v chitosan solution) and LMs-Res-Ch-plus (8.75 % w/v chitosan solution) were prepared by adding a chitosan solution to the formed particles. The mean diameter of the particles were 68.5±3.1 um, 76.3±5.2 um and 84.5±8.1 um for LM-Res, LM-Res-Ch and LM-Res-Ch-plus respectively, suitable for nasal delivery. Chitosan coating changed the particle surface charge from a negative zeta potential value (-12.7±2.1 mV) for the uncoated particles to a higher positive values respectively 24.0±4.7 and 44.6±3.1 mV for the chitosan coated LM-Res-Ch and LM-Res-Ch-plus .Permeation studies across human NCM460 cell monolayers demonstrated that their transepithelial electrical resistance (TEER) values were not modified in the presence of free resveratrol, unloaded LMs, loaded LMs-Res or LMs-Res-Ch. On the other hand, the TEER values decreased from 150±7 to 41±3 Omega.cm2 in the presence of LMs-Res-Ch-plus, which corresponded to a significant increase in the apparent permeability (Papp) of resveratrol from 518±8×10−4 cm/min to 750±98×10−4 cm/min. In vivo studies demonstrated that no resveratrol was detected in the rat cerebrospinal fluid (CSF) after an intravenous infusion of the polyphenol. Conversely the nasal delivery of resveratrol in a chitosan suspension or encapsulated in uncoated LMs-Res dispersed in water achieved the uptake of resveratrol in the CSF with Cmax after 60 min of 1.30±0.30 ug/ml and 0.79±0.15 ug/ml , respectively. However, a dramatic increase in the levels of resveratrol reaching the CSF was attained by the administration of an aqueous suspension of LM-Res-Ch-plus with a Cmax after 60 min of 9.7 ± 1.9 ug/ml. This marked increase in the CSF bioavailability was achieved without any distribution in the systemic circulation, demonstrating a direct and specific nose to brain delivery.

Keywords	resveratrol; lipid microparticles; chitosan-coating; nasal formulation; in vitro permeation studies; brain uptake.
Taxonomy	Bioadhesive, Polysaccharides, Controlled Drug Release

Corresponding Author	Santo Scalia
Corresponding Author's Institution	University of Ferrara
Order of Authors	Valentina Trotta, Barbara Pavan, Luca Ferraro, Sarah Beggiato, Daniela Traini, Larissa Gomes des Reis, Santo Scalia, Alessandro Dalpiaz
Suggested reviewers	Regina Scherließ, Eliana Souto, Shakila Rizwan, William H. Frey II

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EDITORIAL OFFICE
EUROPEAN JOURNAL OF PHARMACEUTICS AND BIOPHARMACEUTICS

Dear Editor,

Please find enclosed the manuscript: **"Brain targeting of resveratrol by nasal administration of chitosan-coated lipid microparticles**" by Valentina Trotta , Barbara Pavan, Luca Ferraro, Sarah Beggiato, Daniela Traini, Larissa Gomes Des Reis, Santo Scalia and Alessandro Dalpiaz , that we would like to submit for publication in the EUROPEAN JOURNAL OF PHARMACEUTICS AND BIOPHARMACEUTICS.

The submitted manuscript comprises original research that has not been published previously and it is not under consideration for publication elsewhere. All authors have read and approved the text and consent to its publication. The authors report no conflicts of interest.

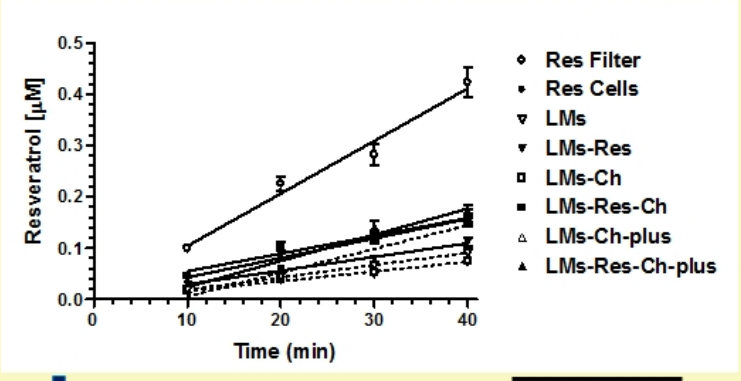
The submitted manuscript describes the development of a chitosan-coated lipid microparticle system loaded with the natural neuroprotective agent, resveratrol and designed for its direct nose-to-brain delivery. Although the activity of resveratrol against neurological disorders such as Alzehimer's and Parkinson's diseases has been reported, these pharmacological beneficial effects are compromised *in vivo* and fail to translate into clinical applications, because of resveratrol poor stability, high metabolism and limited bioavailability in the CNS. In order to overcome this problem, the nasal administration of resveratrol incorporated in nanoemulsion and nanosuspension has been previously reported. A new approach is described in the submitted study, based on the nasal delivery of lipid microparticles loaded with resveratrol and coated with chitosan. The developed carrier exhibits several advantages compared to the published systems, including higher biocompatibility, a greater enhancement of resveratrol bioavailability in the rat brain (12 fold increase vs 6-7 fold reported in the previous studies) and especially a very specific delivery of resveratrol in the CNS with no distribution in the systemic circulation (in the previous studies 24-35% of the nasal administered resveratrol was found in the rat blood). To the best of our knowledge, this work represents the first example of the use of lipid microparticles as carrier for the delivery of resveratrol to the CNS via nasal administration. The results reported in the submitted manuscript indicate that the nasal administration of chitosan-coated lipid microparticles represents a promising system to enhance the effect of resveratrol in the treatment of brain disorders.

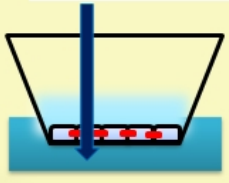
Yours sincerely

Dr. Santo Scalia

Dipartimento di Scienze Chimiche e Farmaceutiche
via Fossato di Mortara, 17-19
44100 Ferrara – Italy ; Phone: +39-0532-455919; E-mail: sls@unife.it

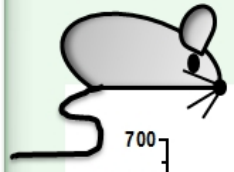
1. Resveratrol (Res) in vitro cell permeability using LMs formulations coated and uncoated with chitosan (Ch)

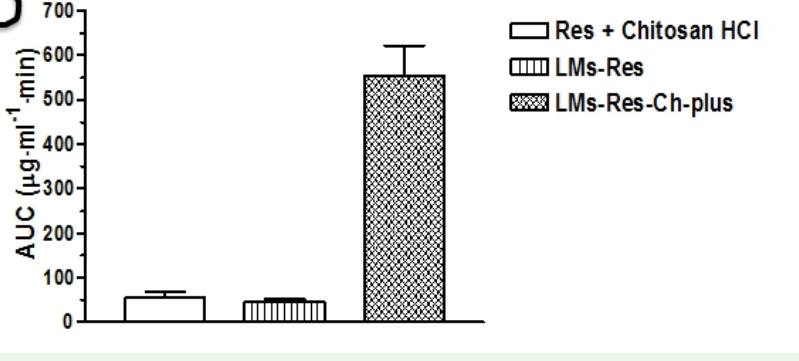




	P_{app} ($\times 10^{-4}$ cm/min)
LMs-Res	390 ± 20
LMs-Res-Ch	510 ± 80
LMs-Res-Ch-plus	750 ± 98

2. Res in cerebrospinal fluid after in vivo nasal administration of LMs formulations coated and uncoated with chitosan





Brain targeting of resveratrol by nasal administration of chitosan-coated lipid microparticles

Valentina Trotta [a], Barbara Pavan[b], Luca Ferraro[b], Sarah Beggiato[b], Daniela Traini[c], Larissa Gomes Des Reis[c], Santo Scalia[a,*] and Alessandro Dalpiaz [a]

[a] Department of Chemical and Pharmaceutical Sciences, University of Ferrara, Ferrara, Italy

b Department of Life Sciences and Biotechnology, University of Ferrara, Ferrara, Italy

[c] Discipline of Pharmacology, School of Medicine, University of Sydney and Woolcock Institute of Medicinal Research, Sydney, Australia

* Corresponding author :

Dr. Santo Scalia

Dipartimento di Scienze Chimiche e Farmaceutiche

Via Fossato di Mortara, 17

44121 Ferrara

e-mail: sls@unife.it

tel: +39-0532-455919

ABSTRACT

Lipid microparticles (LMs) uncoated or coated with chitosan and containing the neuroprotective polyphenol, resveratrol were developed for its targeting to the brain via nasal administration. The lipid microparticles loaded with resveratrol (LMs-Res) were produced by melt emulsification, using stearic acid as lipid material and phosphatidylcholine as the surfactant. The chitosan coated particles LMs-Res-Ch (1.75% w/v chitosan solution) and LMs-Res-Ch-plus (8.75 % w/v chitosan solution) were prepared by adding a chitosan solution to the formed particles. The mean diameter of the particles were 68.5±3.1 μm, 76.3±5.2 μm and 84.5±8.1 μm for LMs-Res, LMs-Res-Ch and LMs-Res-Ch-plus respectively, suitable for nasal delivery. Chitosan coating changed the particle surface charge from a negative zeta potential value (-12.7±2.1 mV) for the uncoated particles to a higher positive values respectively, 24.0±4.7 and 44.6±3.1 mV for the chitosan coated LM-Res-Ch and LM-Res-Ch-plus . Permeation studies across human NCM460 cell monolayers demonstrated that their transepithelial electrical resistance (TEER) values were not modified in the presence of free resveratrol, unloaded LMs, loaded LMs-Res or LMs-Res-Ch. On the other hand, the TEER values decreased from 150±7 to 41±3 $\Omega \cdot cm^2$ in the presence of LMs-Res-Ch-plus, which corresponded to a significant increase in the apparent permeability (P_{app}) of resveratrol from $518 \pm 8 \times 10^{-4}$ cm/min to $750 \pm 98 \times 10^{-4}$ cm/min. In vivo studies demonstrated that no resveratrol was detected in the rat cerebrospinal fluid (CSF) after an intravenous infusion of the polyphenol. Conversely, the nasal delivery of resveratrol in a chitosan suspension or encapsulated in uncoated LMs-Res dispersed in water achieved the uptake of resveratrol in the CSF with C_{max} after 60 min of 1.30±0.30 μg/ml and 0.79±0.15 μg/ml , respectively. However, a dramatic increase in the levels of resveratrol reaching the CSF was attained by the administration of an aqueous suspension of LMs-Res-Ch-plus with a C_{max} after 60 min of 9.7 ± 1.9 μg/ml. This marked increase in the CSF bioavailability was achieved without any

distribution in the systemic circulation, demonstrating a direct and specific nose to brain delivery.

Keywords : resveratrol, lipid microparticles, chitosan-coating, nasal formulation, in vitro permeation studies, brain uptake

1. Introduction

The polyphenol resveratrol is a natural compound, that is found in different types of fruits, vegetables and also in red wine [1]. This polyphenolic compound exists in two isoforms *cis*- and *trans*-resveratrol, the isomer *trans* being more active than the *cis*-form [2].

Resveratrol has attracted the attention of researchers due to its therapeutic effects against various illness, such as cardiovascular diseases [3], diabetes [4], neurodegenerative diseases [5], lung inflammation [6] and cancer [7]. *In vitro* and *in vivo* investigations have pointed out that the beneficial properties of resveratrol are correlate to its anti-inflammatory and anti-oxidant capabilities [8-10].

Resveratrol has been reported to exert the anti-inflammatory activity through several mechanisms that bring to the inhibition of both the transcription and the release of different inflammatory mediators [8,9]. Furthermore, resveratrol has also been shown to protect the cell from the oxidative damage by means of its anti-oxidant capability based on scavenging of free radicals [10].

Specifically for central nervous system diseases, resveratrol has been reported to be effective against neurologic disorders such as Alzehimer's and Parkinson's diseases, brain ischemia and epilepsy [11-14]. Several mechanisms have been advocated for the neuroprotective activity of resveratrol, including its anti-oxidant and anti-inflammatory action [14-16] , stabilization of neuronal mitochondrial function, improvement of cerebral blood flow, protection of dopaminergic neurons, inhibition of formation and aggregation of amyloid-β peptide and activation of sirtuin 1 [13-15,17-19].

However, these pharmacological beneficial effects are difficult to translate into clinical applications because of resveratrol limited chemical stability, poor aqueous solubility (ca. 3 mg/100 mL) and high metabolism [2,20] which limit its *in vivo* bioavailability. To

circumvent this drawback, different strategies have been developed including the use of drug delivery systems such as cyclodextrins, liposomes, nano- and micro-particles [2, 21-23]. In particular, lipid microparticles (LMs) which consist of a natural lipid-based solid core stabilized by a layer of surfactant at the outer shell, exhibit several favourable characteristics as drug carrier including high biocompatibility and biodegradability, low production cost, adequate physico-chemical stability, protection of the incorporated active substance against degradation and modulation of its release [24]. Moreover, due to their micron dimensions LMs have additional advantages compared to liposomes and lipid nanoparticles, including simpler production and characterization methods, reduced amount of required surfactant, higher stability and loading capacity [24,25].

An additional specific problem for the delivery of appropriate therapeutic resveratrol concentrations in the central nervous system (CNS) is the presence of the blood brain barrier (BBB) which is particularly difficult to permeate [13,15,26]. Indeed, even if it is known that resveratrol is able to permeate in the brain from the blood stream in rodents [27] and humans [28], its distribution in the central nervous system appears very poor in comparison to other tissues [29]. Since the brain is connected to the nose via the olfactory portion of the nasal mucosa [18,30-33], the latter region represents an alternative pathway for the direct delivery of active substances to the central nervous system by-passing the BBB. For instance, it has been recently reported that the nasal administration of deferoxamine or insulin can be efficacious against Parkinson's or Alzheimer's diseases, respectively [34,35]. Moreover, the intranasal drug administration route is non-invasive, easy to access and with favourable patient compliance [18,30,32]. In order to enhance the efficacy of nose to brain targeting, the problem of limited residence time due to the rapid mucociliary clearance and relatively low nasal absorption should be addressed. With regard to this, nasal powder preparations offer the advantage of improved residence time on the mucosal surface compare to liquid formulations.

Moreover, the addition of excipients such as bioadhesive carriers and penetration enhancers should further enhance the bioavailability. Among these excipients, chitosan, a cationic polysaccharide of natural origin, has been reported to increase nasal delivery of active drugs due to its mucoadhesive properties [36,37] and ability to promote the reversible opening of cellular tight junctions thereby increasing paracellular transport [36,37].

Aim of this work was the development and characterization of resveratrol-loaded LMs without or with chitosan coating, as system for the delivery of the polyphenol to the CNS through the olfactory region. The influence of the LMs on cell viability, resveratrol transport ad uptake using NCM 460 cells was evaluated. Moreover, *in vivo* studies were performed on rats comparing the intravenous infusion of a resveratrol solution with the nasal administration of free or microencapsulated resveratrol. The time course of resveratrol levels in blood and cerebrospinal fluid were measured after the intravenous and intranasal administration.

2. Materials and methods

2.1. Materials

Resveratrol (*trans*-3,4',5-trihydroxystilbene) was from Fragon Italia (Bologna, Italy). Tristearin, glyceryl behenate and stearic acid were supplied by Sigma Aldrich (Steinheim, Germany), Gattefosse´ (Cedex, France) and Fluka Chemie (Buchs, Switzerland), respectively. The medium-molecular weight chitosan was obtained by Sigma Aldrich (Steinheim, Germany). Phosphatidylcholine and Tween 80 were supplied by Cargill (Hamburg, Germany) and Fluka Chemie (Bucks, Switzerland), respectively. Chitosan hydrochloride was purchased from FMC Biopolymer AS (Drammen, Norway). Bond-Elut C18-, Isolute C18- and Isolute MF C18- silica cartridges (sorbent weight, 200 mg) were from Agilent Technologies

(Waldbronn, Germany) and International Sorbent Technology (Hegoed, UK), respectively. The high-performance liquid chromatographic (HPLC)-grade methanol, acetonitrile and water were obtained from Sigma–Aldrich (Steinheim, Germany).

NCM-460 cells were kindly provided by Dr. Antonio Strillacci, University of Bologna, Italy. The male Sprague−Dawley rats were provided by Charles-River (Milan, Italy).

2.2. High-performance liquid chromatography

High performance liquid chromatography (HPLC) was used in order to quantify resveratrol. The HPLC system was equipped with a Model LabFlow 3000 pump (LabService Analytica, Bologna, Italy), a Model 7125 injection valve with a 10 µL sample loop (Rheodyne, Cotati, CA, USA) and a Model 975-UV variable wavelength UV–vis detector (Jasco, Tokyo, Japan) set at 306 nm. Separations were achieved on a 5-µm Zorbax SB C-18 column (150 x 4.6 mm i.d.; Agilent Technologies) fitted with a guard column (5-µm particles, 4 mm × 2 mm i.d.; Phenomenex, Torrance, CA, USA) and eluted with 65% (v/v) methanol in water containing 0.4% (v/v) acetic acid. Chromatography was performed at ambient temperature at a flow rate of 0.8 mL/min. For the *in vivo* studies, resveratrol analysis in biological fluid was carried out with a mobile phase composed of 40% (v/v) acetonitrile in water containing 0.4% (v/v) acetic acid and 0.75 mM EDTA, to achieve satisfactory resolution between resveratrol and the internal standard (quercetin) peaks. The flow-rate was 0.8 mL/min and the eluent was monitored at 306 nm for resveratrol and at 370 nm for quercetin. Data acquisition and processing were performed with a personal computer using Clarity Lite chromatographic software (DataApex, Prague, Czech Republic). The identities of resveratrol and quercetin (internal standard) peaks were assigned by co-chromatography with the authentic standards.

The quantification of resveratrol was carried out by the peak area correlated with the predetermined standard curve over the range 0.25-100 μg/mL. The calibration curve was linear (n=8, R =0.994, P < 0.001). The chromatographic precision, represented by relative standard deviations (RSD), was evaluated by repeated analysis (n = 6) of the same sample solution containing resveratrol at a concentration of 10 μg/mL. The RSD value was 1.3%.

For CSF simulation, standard aliquots of balanced solution (PBS Dulbecco's without calcium and magnesium) in the presence of 0.45 mg/mL BSA were employed [38,39]. For the assay of resveratrol in CSF, the chromatographic precision was evaluated by repeated analysis (n = 6) of the same sample solution containing 5 μg/mL resveratrol (RSD = 4.0 %) in simulated CSF and calibration curves of peak areas versus concentration were generated in the range 0.1-15 μg/mL (n =6, R =1, P < 0.001).

Resveratrol quantification in rat blood was on the basis of peak area for the ratio resveratrol/quercetin (internal standard). Calibration standards were prepared by spiking blood extracts with the internal standard (quercetin) and with known amounts of resveratrol corresponding to blood concentrations in the range 0.25-20 μg/mL. These solutions were analysed by HPLC and the obtained calibration curves of peak area ratios versus concentrations were linear (n=6, R=0.993, P<0.001). The precision of the method, evaluated by replicate analyses (n= 6) of rat blood extract containing the internal standard (quercetin), and resveratrol at a level of 10 μg/mL, was demonstrated by relative standard deviations < 2.0 %.

2.3. Lipid microparticle preparation

LMs loaded with resveratrol were prepared using the melt oil/water emulsification technique, via a phase inversion procedure (aqueous phase poured into the molten lipid) in

order to avoid loss of both, resveratrol and excipient during the particles preparation procedure.

In brief, the hot aqueous phase (40 mL) in which the surfactant phosphatidylcholine (1.75% w/v) was dispersed, was poured into the melted lipid phase (3.8 g) in which resveratrol (0.35 g) was dispersed. The two phases were mixed using an Ultra Turrax T-25 mixer (IKA-Werk, Staufen, Germany) at 13500 rpm for 1.30 min at a temperature of 75-85 °C. The obtained emulsion was cooled to room temperature using an ice bath under magnetic stirring and then subject to centrifugation at 6000 rpm for 15 min. Finally, the precipitate was lyophilized in order to obtained water-free microparticles.

The LMs coated with chitosan were prepared by adding, under stirring, a chitosan solution (1.75 %, w/v) in aqueous acetic acid (pH 4.2) to the LMs suspension formed during the cooling phase of the emulsion. Throughout the manuscript the LMs loaded with resveratrol, in the absence and in presence of chitosan (Ch) are indicated as LMs-Res and LMs-Res-Ch, respectively. Moreover, a LM formulation loaded with resveratrol and coated with chitosan, using a higher concentration of the polysaccharide in aqueous acetic acid (8.75%, w/v), was also prepared and indicated as LMs-Res-Ch-plus.

Unloaded LMs were also prepared with the same procedures, but omitting resveratrol.

2.4. Particle morphology

The LM morphology was observed using a bench top scanning electron microscopy (SEM) (JEOL JMC-6000 SEM, Tokyo, Japan) at 15 kV. Powders of LMs formulations were randomly placed on the top of carbon sticky tapes that was previously attached on the aluminum stubs. Then the samples were coated with gold for two minutes by the Smart coater (JOEL, Tokyo, Japan).

2.5. Particles size analysis

Laser diffraction (Mastersizer 3000, HYDRO; Malvern, Worcestershire, United Kingdom) was used in order to measure the particle size distribution of LM formulations. The LMs were dispersed in water with 0.25% Tween 80 to obtain a c.a. 0.22 mg/mL particle concentration for each sample and analyzed with a feed pressure of 4 bar, obscuration interval between 0.1 to 15% and a refractive index of 1.762. Analyses were performed in triplicate. Particle surface charge was evaluated by dynamic laser light scattering using a folded capillary zeta cell on a Malvern Nano Series ZS Instrument at 25˚C. Light scattering data were obtained at a detection angle of 173°. Particles were dispersed in filtered (0.2 μm) deionized water. Six measurements were performed for each sample.

2.6. Resveratrol loading

The LMs (10 mg) were dispersed in ethanol (4 mL) in sealed glass vials, heated (80 °C, 5 min) and sonicated for 10 min. The obtained samples were diluted to volume (20 mL) with methanol and filtered using 0.45 μm membrane filters and assayed by HPLC. The polyphenol loading was determined as the percentage ratio between the amount of resveratrol assayed in the LMs and the total weight of the particle sample.

The encapsulation efficiency was calculated as the percentage ratio between the quantity of resveratrol entrapped in the microparticles and the amount of polyphenol added to the melted lipid phase during preparation. The results were the average of three determinations.

2.7. In vitro resveratrol release

Resveratrol dissolution and release from the LMs were evaluated by adding raw resveratrol (ca 0.8 mg) or LMs, containing an equivalent amount of the polyphenol, to 80 mL of phosphate buffer (0.05 M, pH 7.4). The samples were kept under mechanical stirring at 50

rpm and 37 °C. At predetermined time intervals, 1 mL aliquots of the medium were withdrawn and replaced with an equal volume of fresh fluid. The test samples were filtered (0.45 μm) and assayed by HPLC. The resveratrol release (%) was calculated from the total polyphenol content of the LM preparations, which was calculated by extraction of the particles after the release experiment, using the procedure outlined in the previous Section. Data were determined from the average of at least six experiments.

2.8. NCM460 cell culture condition

The NCM460 cell line was grown in DMEM + Glutamax supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere of 95%, with 5% of CO_2. For maximum viability, NCM460 cells were subcultured in fresh and spent growth medium in 1:1 ratio. All cell culture reagents were provided by Invitrogen (Life Technologies, Milan, Italy).

2.9. Differentiation of NCM460 cells to polarized monolayers

Differentiation to NCM460 cell monolayers was performed essentially according to the method reported by Ferretti and co-workers [40]. Briefly, after two passages, confluent NCM460 cells were seeded at a density of 105 cells/mL in a 1:1 ratio of fresh and spent culture medium in 12-well Millicell inserts (Millipore, Milan, Italy) consisting of 1.0 μm pore size polyethylene terephthalate (PET) filter membranes, whose surface was 1.12 cm^2. Filters were presoaked for 24 h with fresh culture medium, and then the upper compartment (apical, A) received 400 μL of the diluted cells, whereas the lower (basolateral, B) received 2 mL of the medium in the absence of cells. Half volume of the culture medium was replaced every 2 days with fresh medium to each of the apical and basolateral compartments. The integrity of the cell monolayers was monitored by measuring the transepithelial electrical resistance

(TEER) by means of a voltmeter (Millicell-ERS; Millipore, Milan, Italy). The measured resistance value was multiplied by the area of the filter to obtain an absolute value of TEER, expressed as $\Omega \cdot cm^2$. The background resistance of blank inserts not plated with cells was around 35 $\Omega \cdot cm^2$ and was deducted from each value. The homogeneity and integrity of the cell monolayer were also monitored by phase contrast microscopy. Based on these parameters, cell monolayers reached confluence and epithelial polarization after 6 days, and monolayers with a TEER stable value around 160 $\Omega \cdot cm^2$ were used for permeation studies. At this point, the medium was replaced with low serum fresh medium (1% FBS) in both the apical and basal compartments.

2.10. Permeation studies across cell monolayers

For permeation studies, inserts were washed twice with pre-warmed PBS buffer in the apical (A, 400 μL) and basolateral (B, 2 mL) compartments, and then PBS buffer containing 5 mM glucose at 37 °C was added to the apical compartment. The apical compartments received the following formulations containing an equivalent amount of resveratrol (0.114 mg/mL): raw resveratrol, LMs-Res, LMs -Res-Ch and LMs-Res-Ch-plus, raw resveratrol with the unloaded microparticles LMs, LMs-Ch and LMs-Ch-plus. During permeation experiments, Millicell inserts loaded with the powder preparations were continuously swirled on an orbital shaker (100 rpm; model 711/CT; ASAL, Cernusco, Milan, Italy) at 37 °C. At programmed time points, the inserts were removed and transferred into the subsequent wells containing fresh PBS, and then basolateral PBS was harvested, filtered through regenerated cellulose filters (0.45 μm), and injected (10 μL) into the HPLC system for the determination of the concentration of resveratrol. At the end of incubation the apical slurries were withdrawn, filtered, and injected into the HPLC system (10 μL). After the withdrawal of apical samples, 400 μL of PBS was inserted in the apical compartments and TEER

measurements were performed. Permeation experiments of free resveratrol were also conducted using cell-free inserts in the same conditions described above. All the values obtained were the mean of three independent experiments. Apparent permeability coefficients (P_{app}) of resveratrol were calculated according to the following equation [41-43] :

$$P_{app} = \frac{\frac{dc}{dt}V_r}{S_A C} \quad (1)$$

where P_{app} is the apparent permeability coefficient in cm/min; dc/dt is the flux of drug across the filters, calculated as the linearly regressed slope through linear data; V_r is the volume in the receiving compartment (basolateral = 2 mL); S_A is the diffusion area (1.13 cm^2); and C is the compound concentration in the donor chamber (apical) detected at 60 min and chosen as approximate apical concentration.

2.11. In vivo resveratrol administration and quantification

Male Wistar rats (200−250 g) anesthetized during the experimental period received a femoral intravenous infusion of 0.2 mg/mL resveratrol dissolved in a medium constituted by 20% (v/v) DMSO and 80% (v/v) physiologic saline, with a rate of 0.2 mL/min for 5 min. At the end of infusion and at fixed time points, blood samples (100 μL) were collected and CSF samples (50 μL) were withdrawn by cisternal puncture method described by van den Berg et al. [44], as previously reported [45]. Briefly, before the experiments the rats were anesthetized and fixed in a stereotaxic apparatus, after shaving of the skin overlying the neck. A needle connected to a syringe by means of polyethylene tubing and filled with sterile filtered water was attached to a holder of the stereotaxic frame. Following the appropriate stereotaxic

coordinates [44], the needle was brought into position to carry out the puncture. Before puncturing, an air bubble was drawn into the needle with the syringe at the other end of the collection tubing. For puncturing, the needle was gently moved through the skin and muscles toward the cisterna magna. During the needle placement, the syringe plunger was pulled back to create negative pressure; thereafter, the needle advancement was continued until the air bubble moved into the tubing followed by CSF. Then, the syringe and the tubing were disconnected from the CSF collection system and a Hamilton syringe was attached to the CSF collection tubing and used for CSF withdrawal. After sampling, the Hamilton syringe was disconnected and the tubing was closed with a clamp. Subsequent samples were taken by removing the clamp from the tubing, followed by attachment of the Hamilton syringe. This procedure requires a single needle stick and allows the collection of serial (40−50 μL) CSF samples which are virtually blood-free. A total volume of about 150 μL of CSF was collected during the experimental session. Considering that the total CSF volume in adult rats ranges from 300 to 400 μL [46,47], 50 μL of CSF should represent about 12% of the original total volume.

Four rats were employed for femoral intravenous infusions. The CSF samples (10 μL) were immediately injected into HPLC system for resveratrol assay. The rat blood samples were subjected to solid-phase extraction using a Isolute MF C18-silica cartridge. To a portion (0.1 mL) of the rat blood, 0.1 mL of the standard solution (100 μM quercetin in water containing 1% DMSO) were added. After mixing, the sample was applied by positive pressure (flow-rate, ca. 1 mL/min) to a pre-conditioned (2 mL of methanol and then 2 mL of water containing 0.75 mM EDTA) Isolute MFC18 cartridge which was washed successively with 4 mL of water containing 0.75 mM EDTA and 2 mL of 15% methanol in water. After light drying by vacuum, the cartridge was eluted with 2 mL of methanol. The latter fraction was reduced to dryness under a nitrogen stream, the residue reconstituted in 150 μL of mobile

phase, subjected to centrifugation (15000 g for 5 min) and a portion (10 μL) of the obtained supernatant injected onto the HPLC column.

For the determination of resveratrol recovery from the blood matrix, rat blood (0.1 mL) was spiked with 0.1 mL of the internal standard solution (100 μM quercetin) and with resveratrol (1 and 10 μg/mL). The samples were processed by solid-phase extraction as outlined above and the recovery was calculated as the percent ratio of the peak areas of resveratrol extracted from the test samples with that obtained by direct injection of an equivalent amount of the analyte dissolved in the mobile phase. The recovery of resveratrol from rat blood was ≥ 80.3%.

The *in vivo* half-life of resveratrol in the blood was calculated by nonlinear regression (exponential decay) of terminal concentration values in the time range between 1h and 6h after infusion and confirmed by linear regression of the log concentration values versus time. Nasal administration of resveratrol was performed on anesthetized rats laid on their backs. An aliquot (50 μL) of an aqueous suspension of resveratrol (2 mg/mL) was introduced in each nostril of the rats using a semiautomatic pipet which was attached to a short polyethylene tubing. The aqueous suspension of resveratrol was obtained in the absence and in the presence of chitosan hydrochloride (7.5 mg/mL), as absorption enhancer. The tubing was inserted approximately 0.6−0.7 cm into each nostril. After the administration, blood (100 μL) and CSF samples (50 μL) were collected at fixed time points and analysed as described above. Four rats were employed for nasal administration of each type of resveratrol suspension. The resveratrol loaded microparticles uncoated (LMs-Res, 37 mg/mL) and coated with chitosan (LMs-Res-Ch-plus, 30.3 mg/mL) were introduced in each rat nostril as a 50 μL aqueous suspension. After administration of the formulations, blood (100 μL) and CSF samples (50 μL) were collected at fixed time points, and analysed as described above. Four rats were employed for nasal administration of the suspension of each type of microparticles. All *in*

vivo experiments were performed in accordance with the European Communities Council Directive of September 2010 (2010/63/EU), the Declaration of Helsinki, and the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health (Bethesda, Maryland). The protocol of all the *in vivo* experiments has been approved by the Local Ethics Committee (University of Ferrara, Ferrara, Italy). Every effort was completed to reduce the number of animals and their suffering. The area under concentration curves of resveratrol in the CSF (AUC, μg mL^{-1} min) were calculated by the trapezoidal method.

2.12. Statistical analysis

Statistical analysis of data were performed by one way ANOVA followed by Bonferroni post-test. $P < 0.05$ was considered statistically significant. All the calculations were performed by using the computer program Graph Pad Prism (GraphPad Software Incorporated, La Jolla, CA, USA), which was employed also for the linear regression of the cumulative amounts of the compounds in the basolateral compartments of the Millicell systems. The quality of fit was determined by evaluating the correlation coefficients (R) and P values.

3. Results and discussion

3.1. Lipid microparticle preparation and characterization

In order to produce an inhalable resveratrol powder formulation for nasal delivery, LM based systems were used as the polyphenol carrier. For this study, a melt emulsification technique was employed to manufacture the LMs loaded with resveratrol since it avoids the

use of organic solvents. Moreover, this technique has been previously utilized to prepare LMs for in vitro nasal deposition studies [23].

Various lipids (tristearin, glyceryl behenate and stearic acid) in combination with phosphatidylcholine as biocompatible surfactant were evaluated for the preparation of the LMs. The polyphenol loading into the lipid microparticles was 6.8 ± 0.1 %, 5.5 ± 0.4% and 6.6 ± 0.8 % (w/w) corresponding to an entrapment efficiency of 80.6 ± 1.4 %, 65.9 ± 4.2 % and 78.6 ± 8.1 % for LMs manufactured with tristearin, glyceryl behenate and stearic acid, respectively. These data indicated that the type of lipid used influences the microparticle entrapment capacity. This phenomenon is probably due to different affinity between the polyphenol and the examined lipids [25].

The release of resveratrol from the LMs was significantly lower (ANOVA) than its dissolution, thus suggesting the entrapment of resveratrol in the lipid particle matrix (Figure 1). Differences among the LMs based on the various lipids were statistically significant at 60 min (stearic acid-LMs versus tristearin-LMs) and 120 min (stearic acid-LMs versus tristearin-LMs and glyceryl behenate-LMs versus tristearin-LMs). The microparticles prepared with stearic acid (LMs-Res) achieving the highest release rate (Figure 1) were selected for further experimentation, in order to enhance the amount of resveratrol available to the nasal mucosa.

A key factor which limits the effectiveness of products for nasal administration is their rapid removal by mucociliary clearance. In order to prolong the residence time of powder formulations on the nasal mucosal, the addition of excipients with mucoadhesive properties has elicited a considerable interest [36]. Among the commonly used bioadhesive excipients for nasal powder formulations, chitosan exhibits several advantages including biocompatibility and absorption enhancing properties [36,37,48]. Accordingly, LM formulations based on stearic acid and coated with chitosan were manufactured. The coating

was achieved by mixing chitosan solutions at different concentrations (1.75 % or 8.75 %, w/v) with the LM suspension [22] and the obtained preparations were indicated as LMs-Res-Ch (1.75% chitosan solution) and LMs-Res-Ch-plus (8.75% chitosan solution). The resveratrol content of the latter particle formulations was 6.4 ± 0.5% and 6.8± 0.6% (w/w), that corresponded to an entrapment efficiency of 76.5% and 81.0%, for LMs-Res-Ch and LMs-Res-Ch-plus, respectively. These results indicated that coating of the particles with chitosan did not significantly affect the polyphenol loading of the LMs, in comparison with the uncoated lipid particles.

Release studies performed on the microparticles coated with chitosan indicated that, with the exception of the values at 15 min, there was not significant difference (ANOVA) compared to the curve measured for the uncoated LM-Res (profiles not shown). SEM analysis of the stearic acid-based LM-Res, LM-Res-Ch and LM-Res-Ch-plus revealed an irregular shape with a rough surface due to the presence of fragments which increased for the LM-Res-Ch and LM-Res-Ch-plus systems (Figure 2). The SEM micrographs indicated for all LM systems a size much greater than 20 μm and hence suitable for nasal delivery [49]. This dimensional evaluation was confirmed by laser diffraction analysis which gave a volume median diameter of 68.5 ±3.1 μm, 76.3±5.2 μm and 84.5±8.1 μm for LM-Res, LM-Res-Ch and LM-Res-Ch-plus, respectively. Zeta potential measurements indicated a negative value (-12.7±2.1 mV) for the uncoated particles whereas the chitosan-coated LM-Res-Ch and LM-Res-Ch-plus exhibited positive zeta potential values which increased with the increase of the chitosan concentration used for their preparation, respectively 24.0±4.7 and 44.6±3.1 mV. The change of the surface charge from a negative value to a positive values following coating of the lipid particles demonstrated the efficient adsorption of the cationic biopolymer on the particle surface. Moreover, the marked increase in the absolute zeta potential value for the coated particles should ensure a higher stability for these systems.

3.2. Permeation studies

The cells of the olfactory epithelium in the nasal cavity are tightly connected by "tight junctions", constituted by a series of membrane proteins interacting with components of the cytoskeleton [48]. In order to evaluate the potential influence of the LMs without or with chitosan coating on the permeability properties of resveratrol, we have performed *in vitro* permeation experiments across monolayers of cells strictly connected by tight junctions. Unfortunately, the RPMI 2650 cell line, derived from a human nasal anaplastic squamous cell carcinoma of the nasal septum, was unsuitable for the evaluation of nasal transport, being unable to form a tight cell layer, a phenomenon caused by poor differentiation and lack of polarization of cells [50,51].

As a consequence, for permeation studies, the normal human colon mucosal epithelial cell line NCM460 [52] was chosen as an *in vitro* epithelial model, being this type of cells able to create monolayers tightly connected, as evidenced by TEER values of about 160 $\Omega \cdot cm^2$. Similar studies have been previously performed with Caco-2 cells [53], however in the present investigation the established NCM460 line was selected because in Caco-2 cell line changes in growth characteristics (monolayers/multilayers) are known to occur due to the loss of contact inhibition and polarization in transformed cell [54,55]. Moreover, as NCM460 cells are not of tumor origin, they retain the physiological characteristics of the human epithelia more closely compared to the pathologically transformed cell lines [55,56]. In order to simulate a nasal administration, the powders of raw resveratrol and the microparticulate systems were introduced in the apical compartments of the "Millicell" systems at levels which achieve the drug saturation. The permeation profiles of raw resveratrol across the Millicell filters alone or coated by monolayers obtained by NCM460 cells are reported in Figure 3,

where the cumulative concentrations in the receiving compartments are shown. Figure 3 reports also the permeation profiles of both raw resveratrol in the presence of unloaded microparticles and the drug encapsulated in the different microparticulate systems. The profiles are referred to the transport from the apical to basolateral compartments. The cumulative amounts in the receiving compartments showed a linear profile within 40 min in all cases ($R \geq 0.976$, $P \leq 0.02$) indicating constant permeation conditions within this time range. The apparent permeability coefficients (P_{app}) of resveratrol (Table 1) have been calculated according to eq. (1), on the basis of the resulting slopes of the linear fits and the resveratrol concentrations detected in the apical compartments after 40 min of incubation of the powders (Table 1), chosen as the approximate apical concentrations. A comparison of the P_{app} values of free resveratrol obtained in the presence ($518 \pm 8 \times 10^{-4}$ cm/min) and in the absence ($820 \pm 89 \times 10^{-4}$ cm/min) of NCM460 cell monolayers indicated a significantly lower permeation of the drug in the presence of cells ($P < 0.001$), confirming the validity of the monolayer as an *in vitro* model of a physiologic barrier. This behavior appeared in agreement with the transepithelial electrical resistance (TEER) values (about 160 $\Omega \cdot cm^2$) attributed to the monolayers before their incubation with the powders (Table 1). The presence of the unloaded LMs induced a significant decrease of the resveratrol P_{app} value ($326 \pm 7 \times 10^{-4}$ cm/min) across the monolayer ($P < 0.05$), whereas this phenomenon was not observed with the unloaded microparticles LMs-Ch , coated with chitosan ($P_{app} = 480 \pm 36 \times 10^{-4}$ cm/min). Interestingly, the unloaded microparticles LMs-Ch-plus, obtained in the presence of a chitosan solution of higher concentration, induced a significant increase ($P < 0.05$) of the resveratrol Papp value ($678 \pm 78 \times 10^{-4}$ cm/min). This effect can be attributed to the well-known ability of chitosan to reversibly open the tight junctions between cells [56,57], as demonstrated by the drastic decrease ($P < 0.001$) of the TEER value of the NCM460 monolayer observed during the incubation of resveratrol with LMs-Ch-plus (TEER decreased

from 150 ± 8 to 41 ± 3 $\Omega \cdot cm^2$, Table 1). It is important to remark that the incubation of resveratrol alone, or in the presence of LMs or LMs-Ch unloaded microparticles, did not induce any significant effect on TEER values of the NCM460 monolayer (Table 1). Similarly, the loaded microparticles LMs-Res and LMs-Res-Ch did not influence the TEER values of the monolayer (Table 1), whereas a significant decrease ($P < 0.001$) was observed during incubation of the loaded microparticles, LMs -Res-Ch-plus (TEER decreased from 150 ± 7 to 103 ± 5 $\Omega \cdot cm^2$, Table 1). Among the loaded microparticles, only those (LMs-Res-Ch-plus) which produced a decrease of the NCM460 monolayer TEER value, significantly increase ($P < 0.001$) the resveratrol P_{app} up to 750 ± 98 x 10^{-4} cm/min (Table1). Therefore, this microparticulate system represents a promising strategy for the enhancement of the resveratrol uptake in the central nervous system, following its nasal administration.

3.3. In vivo resveratrol administration

Taking into account that the resveratrol loaded microparticles based on stearic acid (LMs-Res) were characterized by a satisfactory encapsulation efficiency and release properties and that the chitosan-coated LMs-Res-Ch-plus were able to promote the resveratrol permeation across cell monolayers, these microparticulate systems (i.e., LMs-Res and LMs-Res-Ch-plus) were tested for *in vivo* nasal administration of resveratrol in rats, in order to examine the potential uptake of the polyphenol in the CNS. In particular, the nasal administration of resveratrol was performed using the following different formulations: (i) a suspension of raw resveratrol powder in water, chosen as control, (ii) a suspension of raw resveratrol powder in a chitosan hydrochloride solution, (iii) a suspension of LMs-Res microparticles in water and (iv) a suspension of LMs-Res-Ch-plus microparticles in water. The results were compared with those obtained after the intravenous infusion of resveratrol into the rats.

3.4. Intravenous administration of resveratrol

For the assay of resveratrol in rat blood, conventional liquid extraction followed by centrifugation and solvent removal was initially used as sample preparation method [58]. However, with this pre-treatment procedure, a peak interfering with the determination of resveratrol was observed in the UV chromatogram of the blood sample. Moreover, unsatisfactory recoveries (< 10%) were obtained for resveratrol. In order to overcome this drawback, purification methods based on solid-phase extraction techniques were examined. Disposable C18-bonded silica cartridge were selected since they provide the broadest applicability for pre-treatment of biological fluids. Several C18 silica cartridges (Isolute C18, Isolute MF C18 and Bond-Elut C18) were evaluated using rat blood spiked with resveratrol and the internal standard (quercetin). The highest resveratrol recovery rate (> 80.3%) was achieved by the non-endcapped C18-bonded silica sorbent (Isolute MF C18), probably due to enhance analyte retention through the additional interaction associated with the very accessible silanol groups and therefore this cartridge was selected for the extraction of resveratrol from blood specimens. The analysis of rat blood samples following the intravenous infusion of 0.2 mg of resveratrol indicated that the drug concentration in the blood stream decreased over time from a value of about 7.5 μg/mL (Fig. 4) with an apparent first order kinetic, as confirmed by the linearity of the semilogarithmic plot reported in the inset of Figure 4 ($n = 4$, $R = 0.980$, $P < 0.0001$), and a half-life of 129 ± 8 min. This value is comparable with that observed in humans following resveratrol administration [59]. No resveratrol was detected in CSF of rats within 180 min after the end of intravenous infusion of the drug.

3.5. Nasal administration of resveratrol

Following nasal administration of pure resveratrol (0.2 mg) as a water suspension, no detectable amounts of the drug in the rat blood or CSF were observed within 180 min of administration. In contrast, the nasal administration of the same dose of resveratrol suspended in the chitosan hydrochloride solution (7.5 mg/mL), produced detectable amounts of the drug in the CSF of the rats, as reported in Figure 5. In particular, the resveratrol C_{max} was obtained 60 min after nasal administration of the suspension with a value of 1.30 ± 0.30 μg/mL.

The nasal administration of the same amount of resveratrol encapsulated in LMs-Res microparticles produced an effect similar to that achieved by the suspension of resveratrol in chitosan solution: indeed, the resveratrol C_{max} in CSF was obtained 60 min after nasal administration of the LMs-Res suspension with a value of 0.79 ± 0.15 μg/mL. Moreover, in this case resveratrol was also detected at 30 and 120 min though at lower concentrations, respectively 0.020 ± 0.05 μg/mL and 0.15 ± 0.03 μg/mL (Figure 5).

A drastic increase of the polyphenol uptake in the CSF of the rats was obtained by administration of the water suspension of the chitosan-coated LMs-Res-Ch-plus microparticles containing an equivalent amount of resveratrol (0.2 mg). In this case the drug C_{max} in CSF, observed 60 min after nasal administration, reached a value of 9.7 ± 1.9 μg/mL (Figure 5), which was an order of magnitude higher than that obtained after the nasal administration of the uncoated LMs-Res microparticles. Finally, no resveratrol was detected in the bloodstream of rats within 180 min after nasal administration of the suspensions of resveratrol in chitosan hydrochloride solution, LMs-Res or LMs-Res-Ch-plus microparticles in water.

The areas under the concentration (AUC) curve values (mean ± SD) obtained for resveratrol in the rat CSF after the nasal administration of the drug suspension in the chitosan hydrochloride solution, the LMs-Res and the LMs-Res-Ch-plus microparticles were 54 ± 13,

45 ± 5 and 554 ± 67 μg·mL·min^{-1}, respectively (Figure 6). The ratio between the AUC values of LMs-Res-Ch-plus and those of resveratrol or LMs-Res formulations were 10.2 or 12.3, respectively, indicating the superior ability of the chitosan coated microparticles to induce the resveratrol uptake in CSF of rats after nasal administration, with respect to the other two formulations tested ($P < 0.001$).

The markedly higher levels of resveratrol reaching the brain attained by the LMs-Res-Ch-plus formulation can be ascribed to enhance polyphenol absorption through the mucosal tissue due to chitosan induced reversible opening of the tight junctions in the cell membranes of the nasal epithelium [56], as also suggested by the in vitro permeation studies on the NCM460 cell line. The observed C_{max} values at 60 min (Figure 5) suggests that resveratrol can reach the CNS by paracellular diffusion across the olfactory mucosa, rather than by the transneuronal pathway which is known to require at least 24 hours [60]. In addition, the mucoadhesive nature of the chitosan-coated microparticles should delay the mucociliary clearance prolonging the retention of the formulation on the nasal cavity. The obtained data indicate that the concentration of the chitosan coating of the LMs is critical for efficient brain targeting of resveratrol following nasal administration, since the marked enhancement in CSF bioavailability was achieved only the LMs-Res-Ch-plus system, coated with the chitosan solution at higher concentration (8.75%, w/v). Moreover, the amount of resveratrol measured in the rat brain following administration of the LMs-Res-Ch-plus formulation is comparable and higher than the level which has been shown to elicit *in vivo* neuroprotective activity [19]. Nasal resveratrol formulations based on nanoemulsions and nanosuspensions have been recently described by other authors [18,19,61]. Although a significant increase (about 3-7 fold increase) in the polyphenol brain bioavailability was achieved compared to conventional administrations, a partial distribution (about 24-35%) of the polyphenol in the systemic circulation was observed [18,19,61]. Conversely, the LM system developed here attained

complete targeting of resveratrol to the brain, being resveratrol undetectable in the bloodstream of rats after the nasal administration of the chitosan-coated resveratrol-loaded lipid microparticles (LMs-Res-Ch-plus). The high selectivity achieved by microparticle system developed in this study, should reduce the amount of drug required for the expected pharmacological effect.

4. Conclusions

Resveratrol-loaded LMs uncoated and coated with chitosan were developed in this study and evaluated in vitro and *in vivo* as a carrier system to enhance the targeting of resveratrol to the brain via nasal administration. To the best of our knowledge, the effect of microparticulate powder carrier on the *in vivo* nose to brain uptake of resveratrol has not been reported before. Advantages of the LMs system described here include biodegradability of their components, specific brain delivery, ease of administration as well as non-invasiveness. Pharmacokinetic studies indicated that the intranasal administration of the LMs-Res-Ch-plus particles produced a marked increase in the bioavailability of resveratrol in the brain which should enhance its neuroprotective effect for the treatment of neurological disorders.

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Declaration of interest

The authors declare no conflicts of interest.

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Caption of Figures

Figure 1. Resveratrol dissolution and release profiles from LMs. Values are means± SD (n=5).

Figure 2. SEM micrographs of (A)LMs-Res, (B) LMs-Res-Ch and (C) LMs-Res-Ch plus

Figure 3. Permeation kinetics of resveratrol after introduction in the "Millicell" apical compartments of powders composed by resveratrol in the absence (Res) or in the presence of unloaded microparticles (LMs, LMs-Ch, LMs-Ch-plus) and by the loaded microparticles (LMs-Res, LMs-Res-Ch, LMs-Res-Ch-plus). The permeations were analyzed across monolayers obtained by NCM460 cells. The permeation of free resveratrol was analyzed across the Millicell filters alone (filter) or coated by monolayers (cells). The cumulative amounts in the basolateral receiving compartments were linear within 60 min ($R \geq 0.976$, $P \leq 0.02$). The resulting slopes of the linear fits were used for the calculation of permeability coefficients (P_{app}). All data are reported as mean ± SD of four independent experiments.

Figure 4. Elimination profile of resveratrol after 0.20 mg infusion to rats. The elimination followed an apparent first order kinetic 60 min after the end of infusion, confirmed by the semilogarithmic plot reported in the inset ($n = 4$, $r = 0.980$, $P < 0.0001$). The half-life of resveratrol was calculated to be 129 ± 8 min. All data reported in the figure are expressed as the mean ± SD of four independent experiments.

Figure 5. Resveratrol (Res) concentrations (μg/mL) detected in the CSF after the nasal administration of suspensions of powders constituted by the mixture of the drug and chitosan

HCl and the loaded microparticles (LMs-Res and LMs-Res-Ch-plus). Each dose contained 200 µg of resveratrol. The data are expressed as the mean ± SD of four independent experiments.

Figure 6. Comparison of the AUC values obtained in the CSF of rats after the nasal administration of 200 µg of resveratrol (Res) using different suspension of powders constituted by the mixture of drug with chitosan and the loaded microparticles (LMs-Res and LMs-Res-Ch-plus). The data are expressed as the mean ± SD of four independent experiments. (*P < 0.001 *versus* Res + Chitosan HCl or *versus* LMs-Res)

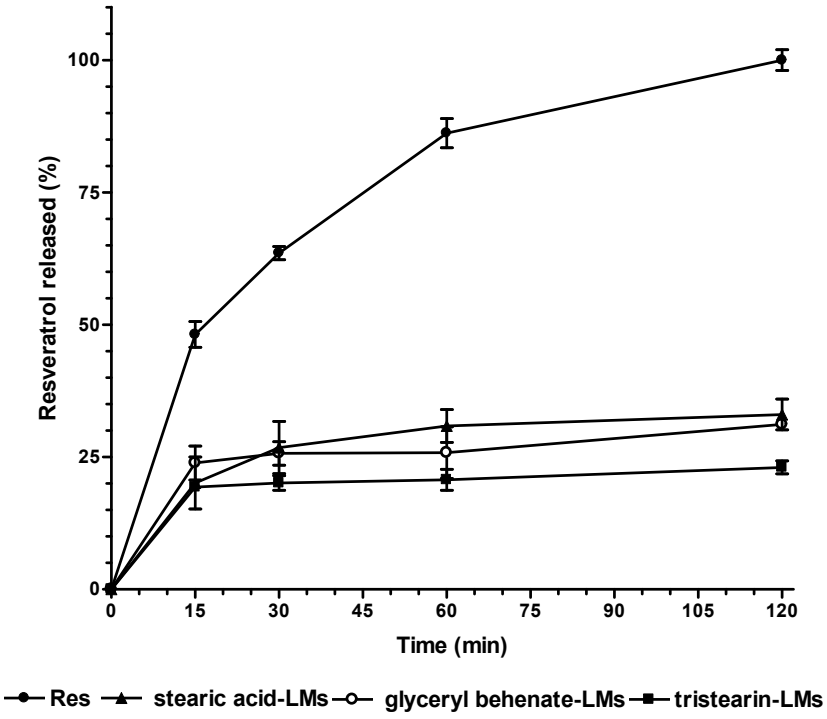
Resveratrol released (%)
100
75
50
25
0
0
15
30
45
60
75
90
105
120
Time (min)
Res
stearic acid-LMs
glyceryl behenate-LMs
tristearin-LMs

Figure 1

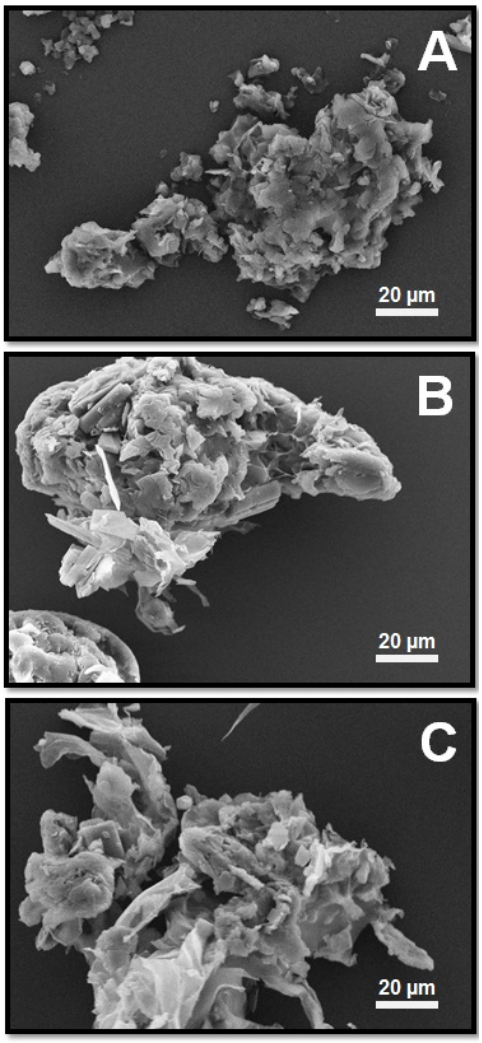

Figure 2

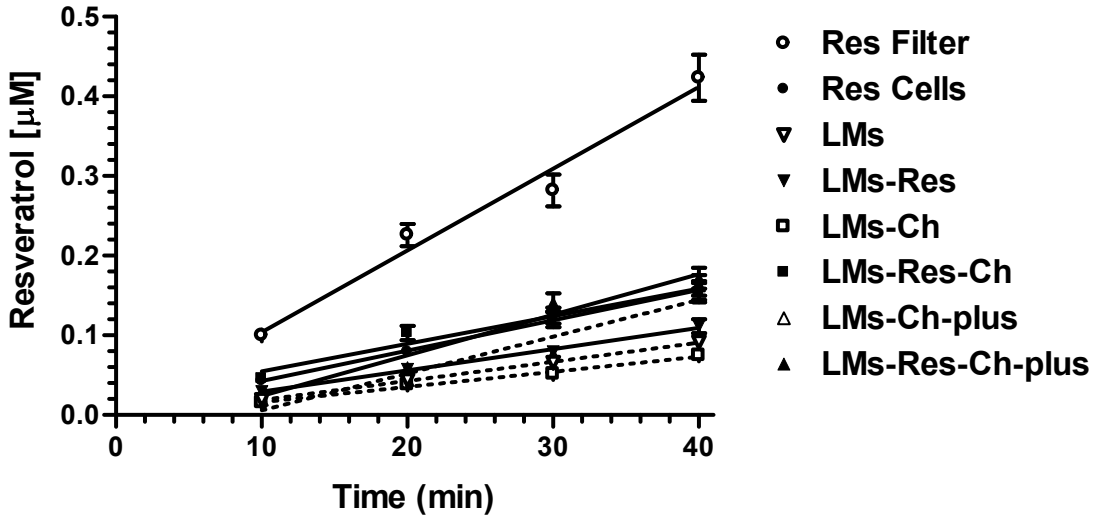
Resveratrol [µM]
0.5
0.4
0.3
0.2
0.1
0.0
0
10
20
30
40
Time (min)
Res Filter
Res Cells
LMs
LMs-Res
LMs-Ch
LMs-Res-Ch
LMs-Ch-plus
LMs-Res-Ch-plus

Figure 3

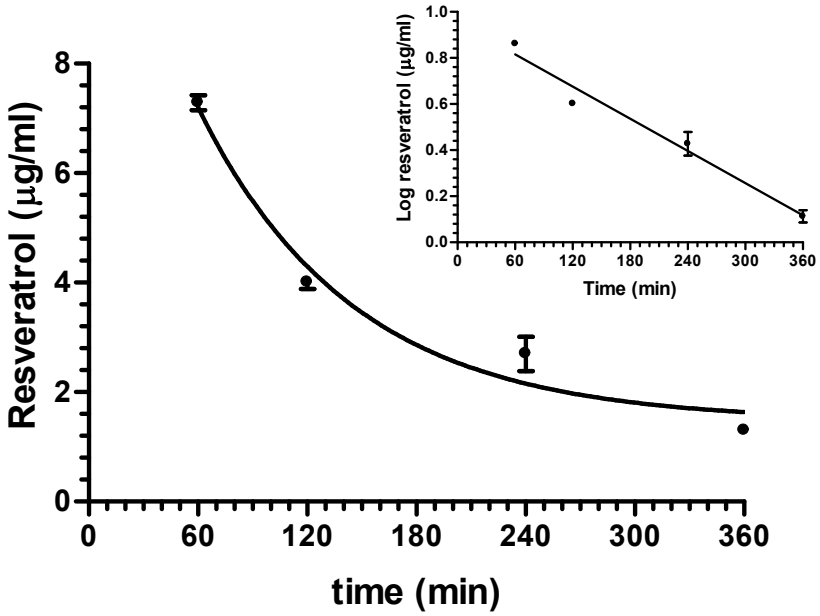
Resveratrol (µg/ml)
time (min)
0
2
4
6
8
0
60
120
180
240
300
360
Log resveratrol (µg/ml)
Time (min)
0.0
0.2
0.4
0.6
0.8
1.0

Figure 4

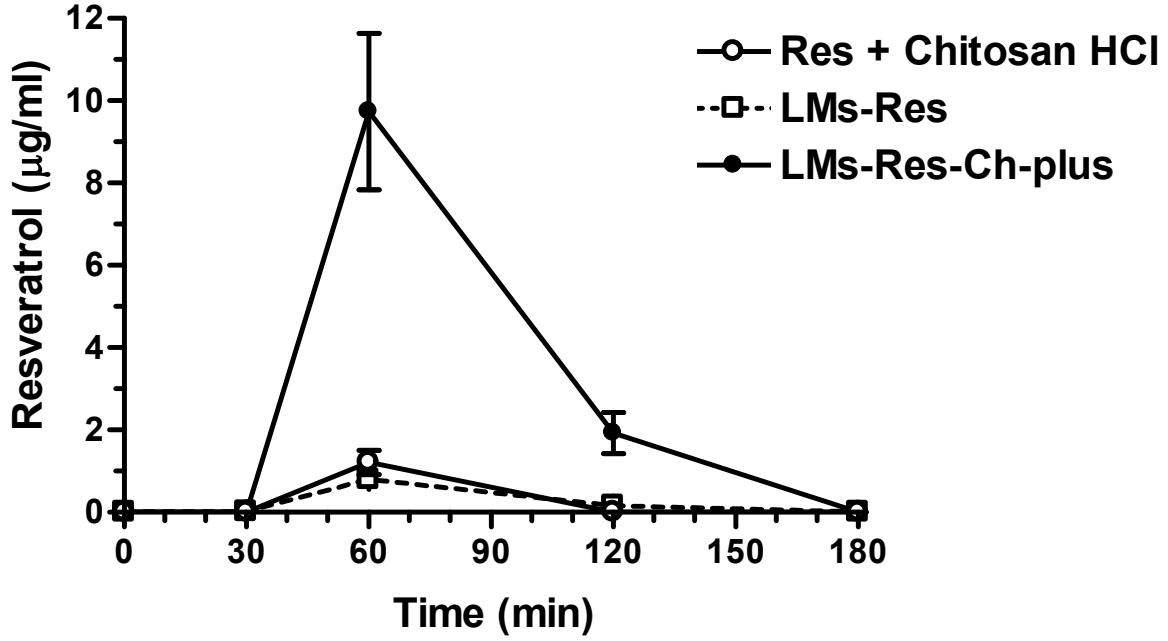
Resveratrol (μg/ml)
12
10
8
6
4
2
0
0
30
60
90
120
150
180
Time (min)
Res + Chitosan HCl
LMs-Res
LMs-Res-Ch-plus

Figure 5

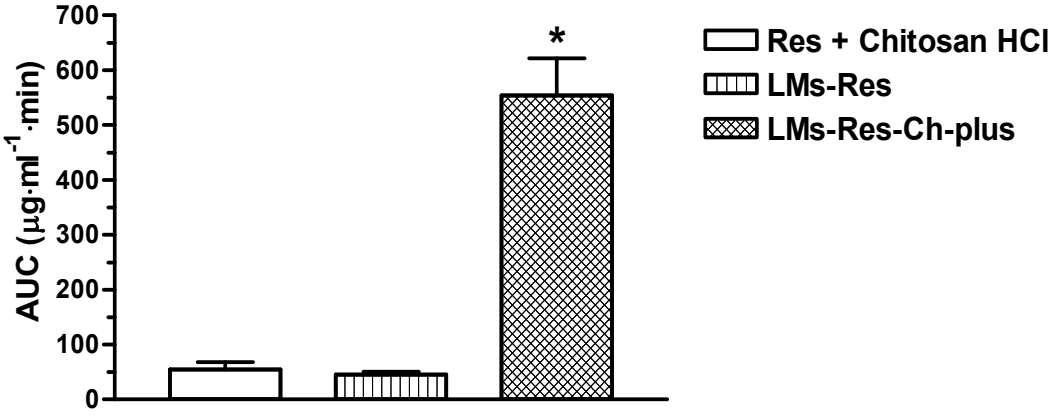
700
600
500
400
300
200
100
0
AUC (μg·ml-1·min)
*
Res + Chitosan HCl
LMs-Res
LMs-Res-Ch-plus

Figure 6

Table 1. Resveratrol permeation data obtained on "Millicell" filters alone (filter) or coated by NCM460 cell monolayers (cells). Resveratrol was introduced in the donor compartment both as raw powder, in the presence of unloaded microparticles or encapsulated in the microparticles. The resveratrol apical concentrations detected at the end of incubation (40 min) were employed for the calculation of the apparent permeation coefficients (P_{app}). The transepithelial electrical resistance (TEER) values were measured before (0 min) and at the end of incubation (40 min). All data related to permeation studies are reported as the mean ± SD of four independent experiments.

				TEER ($\Omega \cdot cm^2$)	
Powder	**Permeation condition**	**apical conc. at 40 min (μM)**	**P_{app} ($\times 10^{-4}$ cm/min)**	**0 min**	**40 min**
Resveratrol	cells	0.134 ± 0,008	518 ± 8	150 ± 7	152 ± 8
Resveratrol+LMs	cells	0.133 ± 0.007	326 ± 7*	159 ± 8	166 ± 9
LMs-Res	cells	0.125 ± 0.007	390 ± 20	161 ± 9	148 ± 7
Resveratrol+LMs-Ch	cells	0.074 ± 0.004	480 ± 36	150 ± 8	152 ± 8
LMs-Res-Ch	cells	0.128 ± 0.008	510 ± 80	152 ± 9	148 ± 8
Resveratrol+LMs-Ch-plus	cells	0.126 ± 0.007	678 ± 78*	150 ± 8	41 ± 3***
LMs-Res-Ch-plus	cells	0.121 ± 0.007	750 ± 98**	150 ± 7	103 ± 5***
Resveratrol	filter	0.219 ± 0.011	820 ± 89**	--	--

*P < 0.05 *versus* Resveratrol (cells) P_{app} value; **P < 0.001 *versus* Resveratrol (cells) P_{app} value; ***P < 0.001 *versus* TEER at "time 0" (0 min).