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Ethosomes for the delivery of anti-HSV-1 molecules: preparation, characterization and *in vitro* activity

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This paper describes the production, characterization and *in vitro* activity of ethosomes containing two molecules with antiviral activity, such as acyclovir (ACY) and N^1-β-D-ribofuranosyl-pyrazole [3,4-*d*]pyridazin-7(6p-chlorine-phenyl)-one nucleoside (N^1CP). Ethosomes were prepared and morphologically characterized by Cryo-TEM. The encapsulation efficiency was 92.3 ± 2.5 % for ACY and 94.2 ± 2.8 % for N^1CP. The release of the drug from vesicles, determined by a Franz cell method, indicated that both drugs were released in a controlled manner. In order to possibly guarantee the stability during long-term storage ethosome suspensions was freeze-dried. It was found that the freeze-dried ethosomes' cakes were compact, glassy characterized by low density and quick re-hydration. However, the storage time slightly influences the percentage of drug encapsulation within ethosomes showing a drug leakage after re-hydration around 10 %. The antiviral activity against HSV-1 of both drugs was tested by plaque reduction assay in monolayer cultures of Vero cells. Data showed that ethosomes allowed a reduction of the ED_{50} of N^1CP evidencing an increase of its antiviral activity. However, ACY remains more active than N^1CP. No differences are appreciable between drug-containing ethosomes before and after freeze-drying. Taken together these results, ethosomal formulation could be possibly proposed as mean for topical administration of anti-herpetic molecules.

1. Introduction

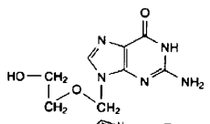
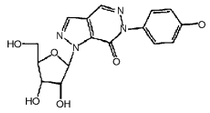
Among drugs able to act against herpes viruses, acyclovir (ACY) is considered the safest and most efficacious. ACY, {9-[(2-hydroxyethoxy)methyl]guanine}, is a synthetic purine nucleoside analogue derived from guanine. The mechanism of action of ACY is related to the inhibition of the HSV DNA replication. ACY can be administered orally or intravenously (in acute cases) for treating herpes virus infections (Modiano et al. 1995; Reichman et al. 1984). The anti-viral drug ACY is also widely used topically for treatment of herpes labialis (Richards et al. 1982; Touitou and Godin 2007). The conventional ACY external formulation is associated with poor skin penetration of ACY to dermal layer resulting in weak therapeutic efficiency (Touitou and Godin 2007; Touitou 2002; Touitou et al. 2000, 2001; Godin and Touitou 2003, Freeman et al. 1986; Jiang et al. 1998; Klimowicz et al. 2007).

To overcome the problem associated with conventional topical preparation of ACY, a number of vesicular delivery systems, such as liposomes, niosomes, ethosomes etc, have been developed. Moreover, due to their role in modeling biological membranes, in the transport and targeting of active agents and also in delaying the drug elimination of rapidly metabolizable drugs, vesicles would be the vehicle of choice (Bando et al. 1996; Abla et al. 2006; Ita et al. 2007) for this purpose. For instance, Horwitz et al. (1999) proposed ACY-containing ethosomes for dermal delivery demonstrating an improved clinical efficacy of the vesicular preparation as compared to that of Zovirax® cream in the treatment of recurrent herpes labialis.

Ethosomes are lamellar vesicles composed of phospholipid (soy phosphatidylcholine), ethanol, and water. They could be defined as lipid vesicular systems embodying ethanol in relatively high concentrations (Touitou and Godin 2007; Touitou 2002). It is thought that ethosomes enhance permeability by fusion of the ethosome with skin lipids, and drug is released at various points along the penetration pathway, perhaps via a follicular transport mechanism (Godin and Touitou 2003; Jain et al. 2007). The clinical evaluation of ACY-containing ethosomes for dermal delivery gave rise to a significant improvement of the disorder after treatment with ethosomal formulation in comparison to a marketed formulation (Zovirax®, Glaxo). The observed clinical progress could be associated with the efficient delivery of the drug into the deep skin strata and production of ACY skin reservoir at therapeutic levels.

Moreover, in order to find drugs able to possibly replace ACY, in the last years many acyclovir-related nucleosides have been synthesized (Baraldi et al. 1998). In particular, a series of 6-substituted N^1- and N^2-β-D-ribofuranosyl-pyrazole[3,4-*d*]pyridazin-7(6*H*)-one derivatives has been prepared to evaluate the drug structure-activity relationship in order to ameliorate the antiviral activity of a many derivatives previously studied by the same authors. The studied compounds were preliminary tested for their *in vitro* antiviral activity against HSV-1. Among them the two *p*-chlorine-phenyl derivatives were found more active

Table 1: Chemical structure and some physicochemical characteristics of the drugs used in the present study

drug	structure	MW	λ_{max} (nm)	logP	ED_{50} (μM)
ACY		209.2	253	−1.76	0.1
N^1CP		378.8	224	0.23	25

ACY: acyclovir; N^1CP: N^1-β-D-ribofuranosyl-pyrazole[3,4-d]pyridazin-7(6*p*-chlorine-phenyl)-one

than those belonging to the parent series; on the other hand, these compounds were less active than ACY (Baraldi et al. 1998).
Taken into account these results, one aim of the present paper was to verify if the inclusion of ACY and/or the N^1-β-D-ribofuranosyl-pyrazole[3,4-*d*]pyridazin-7(6*p*-chlorine-phenyl)-one (N^1CP) (see Table 1 for structures) within ethosomes could influence the *in vitro* antiviral activity.
In particular this report describes: (a) the production and characterization of ethosomes as vehicles of the drugs above mentioned; (b) the determination of drug encapsulation and the release from ethosomal dispersions; (c) the effect of freeze-drying on ethosomes' characteristics and (d) the *in vitro* evaluation of antiviral activity of drug-containing ethosomes before and after freeze-drying.

2. Investigations, results and discussion

2.1. Production and characterization of ethosomes

Ethosomes were spontaneously produced as detailed in the experimental section. Ethosomal suspensions were composed of PC 5 %, ethanol 20 %, drug and isotonic Palitzsch buffer (IPB) to 100 % (w/w). During preparation, dispersions displayed initial optical transparency due to the high ethanol concentration able to maintain PC in solution. By adding increasing concentrations of IPB buffer, PC molecules reorganize within the system resulting in a turbid ethosomal suspension (Table 1).
As described by Jain (2007), after production, ethosomes were subjected to extrusion in order to homogenize vesicles' size. Extrusion was performed through 400 nm and 200 nm pore size membranes. Table 2 reports the vesicles' mean diameters measured by PCS before and after extrusion. As expected, after extrusion ethosomal vesicles were characterized by monomodal distributions and mean diameters reflecting the size of the membrane pore employed for the extrusion. Moreover, after each extrusion cycle polydispersity indices were lowered, confirming the improvement of size distribution due to the extrusion step.

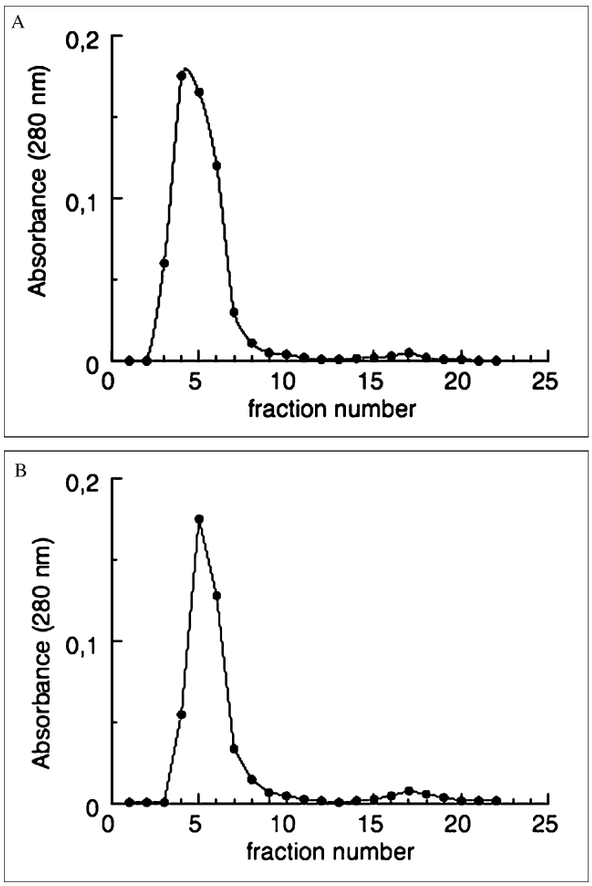


Fig. 1: Gel-permeation chromatography elution profile of ethosomes loaded with ACY (panel A) and N^1-β-D-ribofuranosyl-pyrazole[3,4-*d*]pyridazin-7(6*p*-chlorine-phenyl)-one nucleoside (N^1CP) (panel B)

In order to separate the free drug (namely ACY or N^1CP) from the ethosome-associated drug, a gel-permeation chromatography was performed. The elution profiles reported in Fig. 1 show two peaks with different relative height. The first peak is attributed to the elution of ethosome-associated drug, the second smaller peak is referred to the elution of the free drug. The presence of vesicles in the first peak was indicated by the turbidity of the fraction and by the existence of phospholipids detected by TLC analysis. The presence of the drug in both peaks was proven by HPLC analysis. The encapsulation efficiency of ACY into ethosomes after gel-filtration was found to be 94.2 ± 2.8 %. Ten days after preparation the encapsulation efficiency was 89.6 ± 4.2 % (see Table 2). Concerning N^1CP

Table 2: Average diameter, ζ potential and percentage of drug encapsulation of the produced ethosomes

Drug *treatment*	Mean diameter (nm)	Polyd.§	ζ potential (mV)	% of drug encapsulation (day 1)	% of drug encapsulation (day 10)
ACY					
*not extruded**	943.2 ± 26.3	0.68	/	/	/
*extruded**	257.7 ± 5.1	0.14	−3.15 ± 0.28	94.2 ± 2.8	92.3 ± 2.5
N^1CP					
*not extruded**	1016.4 ± 32.1	0.72	/	/	/
*extruded**	196.9 ± 4.6	0.13	−2.25 ± 0.22	89.6 ± 4.2	86.6 ± 3.9

Data represent the mean of three independent experiments
§ Polyd.: polydispersity
*analyses were performed before and after extrusion. Vesicles were extruded twice through two stacked standard 25 mm diameter polycarbonate filters with 200 nm pore size
ACY: acyclovir; N^1CP: N^1-β-D-ribofuranosyl-pyrazole[3,4-*d*]pyridazin-7(6*p*-chlorine-phenyl)-one

after gel-filtration the encapsulation efficiency was found to be 92.3 ± 2.5 % while 10 days after production was 86.6 ± 3.9 %. In both cases, it is evident that the encapsulation yield was very good and remained high after 10 days due to the lipophilicity of the molecule.

As expected, ζ potentials of both formulations were closed to neutrality (see Table 2).

Fig. 2 shows cryo-TEM images of empty and drug-containing ethosomes. The electron microscopic analysis demonstrates that after extrusion, both empty (panel A) and drug-containing ethosomal suspensions (panels B and C) are constituted by unilamellar vesicles together with a presence of oligo- to multi-lamellar vesicles. However, all the suspensions showed a population quite low polydisperse with an average diameter reflecting the pore size of the membrane used for the extrusion.

2.2. Diffusion experiments

In order to investigate the diffusion of both ACY and N^1CP from the ethosomes, an *in vitro* test based on a Franz cell was employed (Nastruzzi et al. 1993; Esposito et al. 2004). Using the diffusion cell with a synthetic membrane, the method can be utilized to determine the drug diffusion characteristics from the ethosome based formulations and thus can be used as a quality control procedure to assure batch-to-batch uniformity (Shah et al. 1991). Particularly, a cellulose ester membrane with 0.2 μm pore size and phosphate buffer pH 7.4 as receptor phase were used. Throughout the experiments, sink conditions were maintained.

The amount of drug released per unit area (μg/cm^2) was plotted against time. The cumulative amount of both drug released was linear and proportional to the time. The slope, which represents the release rate, steady-state flux, was calculated by linear regression. Correlation coefficients of the regression line were always higher that 0.981 (see Table 3).

It is known that the membrane should not impede or control release rate of drug from the formulation into the receptor phase, acting only as a support (Shah et al. 1991, 1993). This was demonstrated by measuring the diffusion of ACY and N^1CP buffer compared to drug diffusion from ethosomal systems (Fig. 3). The amount of diffused ACY (Fig. 3A) and N^1CP (Fig. 3B) from the solutions were always larger than that from the vesicular systems, suggesting that the membrane employed was not rate limiting. The calculated diffusion coefficients for ACY and N^1CP incorporated within ethosomes are reported in Table 3.

In the case of free solutions, the diffusion coefficients of ACY and N^1CP (J_n values) were, respectively, 3.8 and 4.5-fold higher as compared to the corresponding ethosomal form, thus evidencing the effect of controlled release exerted by the vesicle systems.

2.3. Freeze-drying of ethosomes

Freeze-drying of ethosomes suspensions was investigated in order to explore the stability of the lipids during long-term storage and also the reconstitution of the formulation after re-hydration. Indeed, the target was to obtain vesicles able to maintain the physical and chemical characteristics of the initial formulation as much as possible.

On the basis of our previous research on liposomes (Cortesi et al. 2000), we performed the lyophilization of ethosomes in the presence of sucrose, a cryoprotectant.

Particularly, after production and characterization of ethosomes, the suspension was diluted 1:1 by volume with the sucrose solution and afterwards subjected to freeze-drying for 24 h at a vacuum of 6×10^{-2} mbar.

The obtained freeze-dried ethosome cakes were compact, glassy with low density assuring a very quick re-hydration that results almost instantaneously in a homogeneous aqueous ethosomal suspension. SEM photographs of the freeze-dried cakes showed for both ACY- and N^1CP-containing ethosomes a needlelike structure with a slightly gathered surface (Fig. 4). No appreciable differences in terms of general appearance, re-hydration characteristics, and freeze-dried cake morphology were detectable between empty and drug containing ethosome formulations (data not shown).

After freeze-drying, cakes were re-hydrated with bidistilled water. Table 4 reports the average diameters and size distributions of ethosomes in suspension before and after freeze-drying. As general consideration, the presence of the cryoprotectant induced a decrease of the mean diameter of ethosomes. It should be supposed that the use of sucrose prevents vesicles' aggregation and fusion during the freeze-drying process and leads to a mean size (144 nm) smaller than that of the same vesicular preparation before freeze-drying (197 nm).

In order to evaluate the effect of storage, the freeze-dried ethosomes were re-hydrated after 1 or 10 days from the lyophilization. The results of these experiments indicate that aging has no influence on the re-hydration time of vesicles, being nearly instantaneous, and on vesicles size, being 144.1 nm and 143.4 nm after 1 or 10 days from the lyophilization, respectively. (Fig. 5)

However, the storage time slightly influenced the percentage of drug encapsulation within ethosomes. Particularly, re-hydration after 1 and 10 days from freeze-drying led to a drug leakage from ethosomes of 9.9 ± 3.9 % and 10.8 ± 3.2 % respectively.

2.4. Antiviral activity of drug-containing ethosomes

Safety of the ethosomal carrier was determined by examining Vero cells with the MTT assay. The effect of the cell viability

Table 3: *In vitro* diffusion coefficients of acyclovir and N^1CP determined using Franz-type diffusion cell

Drug *formulation*	R	Fo	Fn	log Fo	log Fn
ACY aqueous solution	0.998	0.19718	0.01885	−0.705	−1.724
ACY ethosome suspension	0.994	0.05189	0.00496	−1.284	−2.304
N^1CP aqueous solution	0.996	0.46059	0.02431	−0.337	−1.614
N^1CP ethosome suspension	0.981	0.10236	0.00540	−0.989	−2.267

Data represent the average of five independent experiments; $p < 0.05$

ACY: acyclovir; N^1CP: N^1-β-D-ribofuranosyl-pyrazole[3,4-d]pyridazin-7(6*p*-chlorine-phenyl)-one

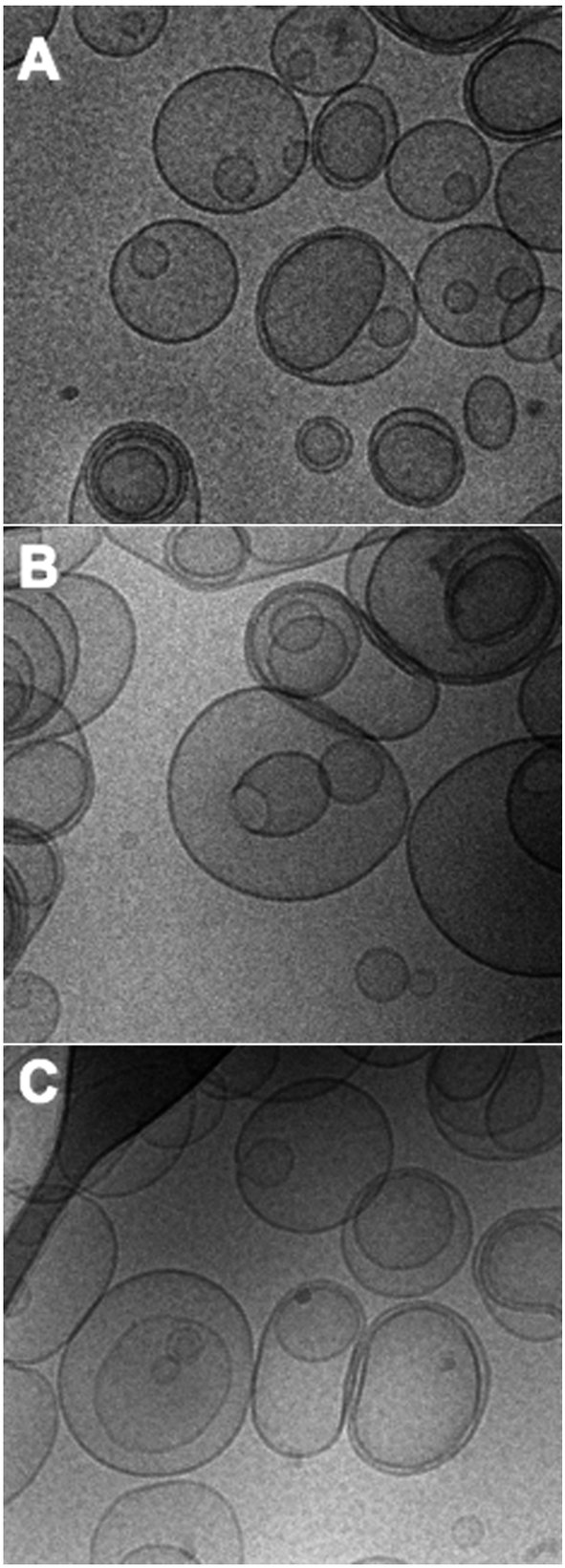


Fig. 2: Cryo-TEM photographs of empty (panel A), ACY-containing (panel B) and N^1CP-containing ethosomes (panel C). Bar represents 100 nm

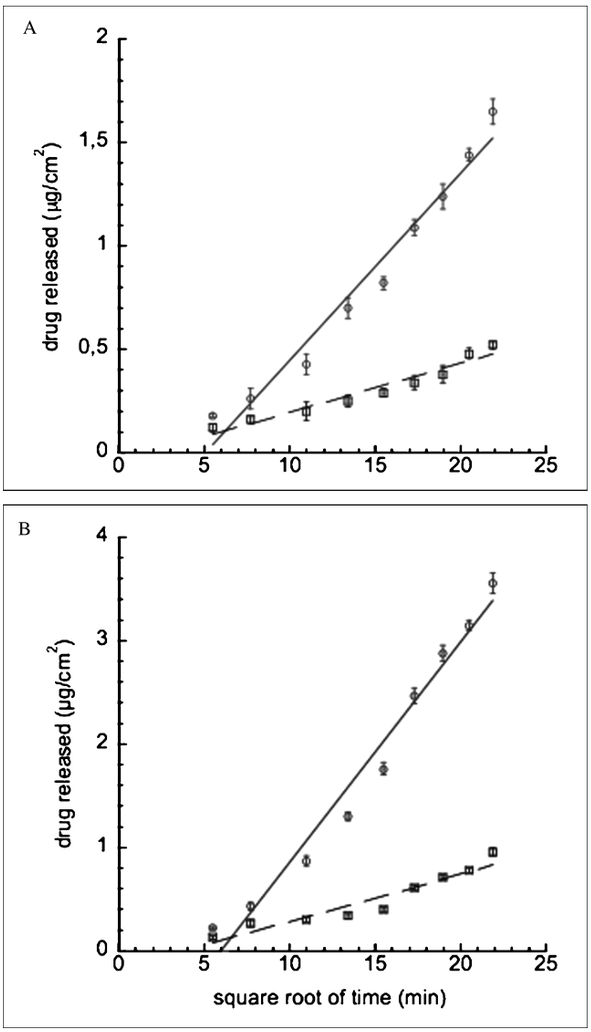


Fig. 3: *In vitro* release rate kinetics from solutions (circle) and ethosome dispersions (square) of ACY (panel A) and N^1CP (panel B). Experiments were performed by a cellulose ester membrane with 0.2 µm pore size and phosphate buffer pH 7.4 as receptor phase. The reported results represent the mean values ± SD of six independent experiments

was tested after a 24 h incubation period. The addition of empty ethosomes suspension resulted in a slight decrease in the cell viability compared with the blank ($p < 0.05$).

The antiviral activity against HSV-1 of drug-containing ethosomes was tested by plaque reduction assay in monolayer cultures of Vero cells (African Green monkey kidney). Several authors indicate Vero cells as a suitable host for HVS-1 (Pope

Table 4: Average diameter and percentage of drug leakage of freeze-dried ethosomes loaded with N^1CP

*N^1CP treatment**	Mean diameter (nm)	Polyd.§	% of drug encapsulation (day 1)	% of drug loss
before	196.9 ± 4.6	0.13	89.6 ± 4.2	0
resuspended after 1 day	144.1 ± 3.7	0.25	79.7 ± 3.9	9.9 ± 3.9
resuspended after 10 days	143.4 ± 2.8	0.22	78.8 ± 3.2	10.8 ± 3.2

Data represent the mean of five independent experiments ±SD; $p < 0.05$
* analyses were performed before freeze-drying and after re-hydration
§ Polyd.: polydispersity
N^1CP: N^1-β-D-ribofuranosyl-pyrazole[3,4-d]pyridazin-7(6*p*-chlorine-phenyl)-one

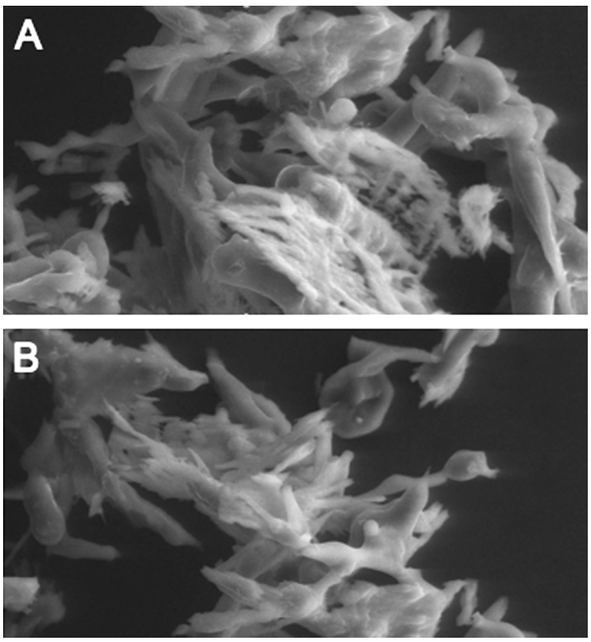


Fig. 4: SEM photographs of freeze-dried cakes of ACY- (panel A) and N^1CP-ethosomes (panel B). As cryoprotectant, sucrose was employed. Magnification was 1.52K X. Bar represents 10 μm

et al. 1998; Walro and Rosenthal 1997). After 1 h of incubation, the virus was removed and then different concentrations of ethosomes (i.e., empty, ACY-containing or N^1CP-containing) were added to the monolayers. As expected, the plaque reduction assay revealed the absence of activity of empty ethosomes in infected Vero cells, at concentrations as high as 1 mM (data not shown). Table 5 shows the ED_{50} values, that is the compound concentration required to reduce the number of plaque, by 50 % of empty or drug loaded ethosomes; in addition the activity of ethosomes after freeze-drying and re-hydration at day 10, was tested. By the analysis of data reported in Table 5, it is evident that N^1CP maintains its lower activity as compared to ACY; however the use of ethosomes allowed a reduction of N^1CP ED_{50} value thus reflecting an increase in antiviral activity. It should be supposed that ethosomes could enhance the penetration of N^1CP within Vero cells suggesting a better biologic effect. The same behavior can be found for re-hydrated N^1CP-ethosomes, it has to be noted that the freeze-drying process does not affect heavily the antiviral activity shown by ethosomes as carrier system. Concerning ACY, the presence of ethosomes causes a slight reduction of the inhibition of virus replication as compared to that displayed by the drug solution. No differences are appreciable between ACY-containing ethosomes before and after freeze-drying.

Taking into consideration the results obtained, it should be supposed that ethosomes could enhance the penetration of N^1CP

Table 5: *In vitro* effect of ACY and N^1CP on plaque reduction

Drug	ED_{50} (μM)		
	free drug	ethosomes before freeze-drying	ethosomes after hydration
ACY	0.12 ± 0.02	0.86 ± 0.31	1.02 ± 0.28
N^1CP	25.31 ± 1.14	14.84 ± 0.43	18.20 ± 0.62

ED50: compound concentration required to reduce by 50 % the number of plaque
Data represent the mean of three independent experiments ±SD; $p < 0.05$

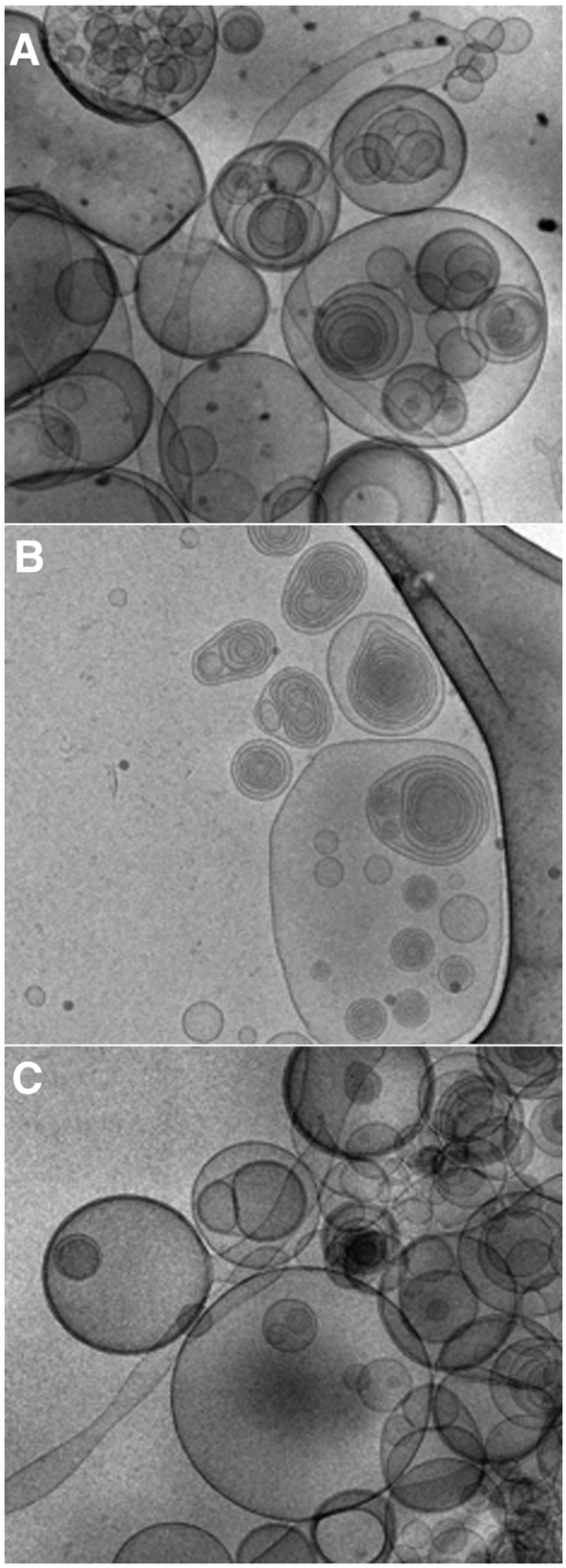


Fig. 5: Cryo-TEM photographs of ethosomes re-hydrated after 10 days from freeze-drying. Panel A: empty vesicles. Panel B: ACY-containing ethosomes. Panel C: N^1CP-containing ethosomes. Bar represents 200 nm

within Vero cells suggesting a better biologic effect. These findings allow us to propose ethosomal formulations as possible means for topical administration of anti-herpetic molecules.

3. Experimentals

3.1. Materials

Soy phosphatidyl choline, Phospholipon® 90G (PC) was purchased from Nattermann Phospholipid GmbH (Cologne, Germany). Absolute ethanol was from Merck KGaA (Darmstadt, Germany), ACY was purchased from Fluka (Bucs, Switzerland). Sucrose was from Roquette (France). All other materials and solvents of the high purity grade were from Sigma Chemical Co. (USA).

3.2. Production of ethosomes

For the preparation of ethosomes PC (10 mg/ml) and drug were dissolved in 2 ml of ethanol at the ratio PC:drug, 10:1 (mol/mol). 8 ml of an aqueous phase constituted of IPB (5 mM $Na_2B_4O_7$, 180 mM H_3BO_3, 18 mM NaCl) was then slowly added to the ethanolic solution under continuous stirring at 700 r.p.m. by an Eurostar digital, IKA Labotechnik, Sardo (Torino, Italy). Mechanical stirring was continued for 5 min at 30 °C.
Homogeneously sized vesicles were obtained by extruding ethosomal dispersions through polycarbonate membranes (Nucleopore Corp., Pleasanton, CA) with pores of calibrated diameter (Mayer et al. 1986) using an Extruder™ (Nipex Biomembranes Inc. Vancouver, Canada). In particular, vesicles were subjected to one extrusion cycle through two stacked 400 nm pore size filters followed by three extrusion cycles through two stacked 200 nm pore size membranes.
In order to separate the free drug from the drug contained within ethosomes, the suspensions were loaded on a gel filtration Sepharose 4B column (Pharmacia, Uppsala, Sweden) (1.5 cm diameter, 50 cm length) pre-equilibrated and eluted with borate buffer. The void volume peak fractions were collected and quantified for vesicle and drug content. Each fraction from the Sepharose 4B column was checked for ACY or N^1CP content by HPLC analysis.
The RP-HPLC analyses were performed with a Jasco apparatus (Jasco, Japan) consisting of a two plungers alternative pump, a variable wavelenght UV-detector, operating at 254 nm for ACY and 220 for N^1CP, and a Rheodyne injection valve mod. 7215 (Cotati, CA, USA) with a 50 µl loop. A Vydac C18 column (25 x 0.46 cm) stainless steel packed with 5 mm particles (Grace, IL, USA) was eluted at room temperature with a mobile phase consisting of 10 % methanol and 90 % NaH_2PO_4 buffer 0.02 M pH3, the flow rate being 0.8 ml/min. Under these conditions the retention time of ACY was 5.32 min and 4.97 min for N^1CP.

3.3. Physical characterization of ethosomes

Cryo-TEM analyses were performed as follows Ethosomal dispersion (2 µl) was placed on a pure thin bar 600-mesh TEM grid, blotted with filter paper and then reduced to a thin film (10–200 nm) spanning the hexagonal holes of the TEM grid. Afterwards, the sample was vitrified by liquid ethane and transferred to a Zeiss EM922 transmission electron microscope for imaging using a cryoholder (CT3500, Gatan Inc., USA). Specimens were examined with doses of about 1000–2000 e/nm2 at 200 kV. Images were recorded digitally by a CCD camera (Ultrascan 1000, Gatan Inc., USA) using a image processing system (GMS 1.4 software, Gatan Inc., USA).
Submicron particle size analysis was performed using a Zetasizer 3000 PCS (Malvern Instr., Malvern, England) equipped with a 5 mW helium neon laser with a wavelength output of 633 nm. Glassware was cleaned of dust by washing with detergent and rinsing twice with water for injections. Measurements were made at 25 °C at an angle of 90°. Data were interpreted using the "method of cumulants".

3.4. In vitro diffusion study

The experiments were carried on using a standard glass Franz release rate cell with 1 cm diameter orifice (0.78 cm^2 area) assembled with a cellulose esters based membrane, 0.2 µm pore size (Schleicher & Schuell, Germany) (Nastruzzi et al. 1993).
As receptor phase a solution of phosphate buffer 0.1 M (pH 7.4) was used. In order to avoid air bubbles formation, the receptor phase was always degassed before use and poured in the cell body to overflowing. To study the drug release rate, 1 ml of drug solution or drug containing-ethosomal suspension was placed into the donor cell compartment and tamped down on the membrane, previously moistened with the receptor phase. The upper part of the chamber was sealed to avoid evaporation. The receptor phase (5 ml) was stirred by a constantly spinning bar magnet at 400 rpm and thermostated at 37 °C. At predetermined time intervals between 1 and 8 h, 0.15 ml of receptor phase were withdrawn and the drug concentration in the receptor phase was measured by HPLC. Each removed sample was replaced with an equal volume of simple receptor phase.
The amount of drug released per unit area (µg/cm^2) was plotted against square root of time. The slope of the line (regression) represents the release rate of the drug. The release rate coefficients were expressed both as experimentally observed fluxes (Fo) and as normalised fluxes (Fn) (Fn = Fo/C, where C is the drug concentration expressed in mg/ml).
The obtained normalised fluxes (Fn) were determined six times in independent experiments and the mean values ± standard deviations were calculated. Statistical analysis was performed by the Student's *t*-test or analysis of variance (ANOVA). The level of significance was taken at *P*-values <0.05.

3.5. Lyophilization of ethosomes

Equal volumes (10 ml) of ethosomes suspension and sucrose solution were mixed together, the ethosome:sucrose ratio being 10:1 by weight. The resulting mixture was rapidly frozen in liquid nitrogen for 10 min and placed at −40 °C on the freeze-dryer, then vacuumed at a pressure of 0.06 mbar for 24 h using a vacuum freeze-dryer Edwards mod. Modulyo (Edwards High Vacuum Inc, Crawley, UK).
The morphology of lyophilized preparations was evaluated by scanning electron microscopy (SEM) observations (360 Stereoscan Cambridge Instruments Ltd, Cambridge, UK).
Re-hydration was performed by adding to the freeze-dried products 20 ml of bidistilled water.

3.6. Cell viability test

Cell viability was determined by a 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoleum bromide (MTT) assay. Vero cells were grown in RPMI 1640 medium (Sigma) supplemented with 10 % fetal calf serum, 1 % λ-glutamine (200 mM) and 1 % mixture of penicillin (100 IU/ml) and streptomycin (100 µg/ml) at 37 °C in a humidified atmosphere containing 5 % CO_2. Vero cells at 2×10^4 cells/well were seeded in 96-well plates. Empty ethosomes (20 µl) were added and incubated for 24 h. Afterwards 50 µl of the MTT solution (50 µg/ml) were added to each well and incubated for 4 h. In order to dissolve the water-insoluble formazan, MTT/medium was removed from each well, and 100 µl of dimethyl sulfoxide (DMSO) was added to each one. The quantity of cell survival was measured with an ELISA assay reader (FLx800, BIO-TEK, Burlingame, CA, USA) at 540 nm.

3.7. Evaluation of antiviral activity

Antiviral activity against HSV-1 by plaque reduction assay was performed according to the standard method described by Hill et al. (1991). 5×10^5 Vero cells/well were seeded in 12-well plates and incubated at 37 °C and 5 % CO_2. When the cells reached 95 % of confluence, they were infected with 100 pfu of HVS-1. After incubation for 1 h at 37 °C to allow viral adsorption, the plates were washed and the medium replaced with maintenance medium containing different concentrations of empty or drug-containing ethosomes (i.e., 50, 33, 25, 16.6, 8.3, 5 µM). After 48 h incubation, the medium was removed and the monolayers were mixed with 0.2 % of γ-globulin in order to prevent the formation of secondary plaques. Afterwards cells were fixed with acetone–methanol (50:50) at 4 °C and stained with a 1 % solution of crystal violet. The number of plaques was counted under a light microscope. The antiviral activity was evaluated as plaque reduction with respect to control cells following the equation:

$$\% \text{ plaque reduction} = (1 - Ps/Pc) \times 100 \quad (1)$$

where Ps is the number of plaque obtained after cell infection and treated with the drug (in the free or microencapsulated form) and Pc is the number of plaque obtained after cell infection and untreated with the drug (control cells).
Data are expressed as mean ± S.D. Statistical analysis was performed by the Student's *t*-test or analysis of variance (ANOVA). The level of significance was taken at *P*-values <0.05.

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