**Enhanced antiviral activity of acyclovir loaded into nanoparticles.**

# Enhanced antiviral activity of acyclovir loaded into nanoparticles

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## Abstract

The activity of antivirals can be enhanced by their incorporation in nanoparticulate delivery systems. Peculiar polymeric nanoparticles, based on a β-cyclodextrin-poly(4-acryloylmorpholine) mono-conjugate (β-CD-PACM), are proposed as acyclovir carriers.

The experimental procedure necessary to obtain the acyclovir-loaded nanoparticles using the solvent displacement preparation method will be described in this chapter. Fluorescent labelled nanoparticles are also prepared using the same method for cellular trafficking studies.

The biocompatibility assays necessary to obtain safe nanoparticles are also reported.

The last section of the chapter describes the assessment of the antiviral activity of the acyclovir-loaded nanoparticles.

## Introduction

Herpes simplex viruses (HSV) type 1 and 2 (HSV-1 and HSV-2) are closely related pathogens of the Herpesviridae family of DNA viruses. Both cause a lifelong, latent infection for which there is no cure or available effective vaccine. HSV-1 is usually transmitted via non-sexual contact and is generally clinically associated with oro-labial infection, whereas HSV-2 is typically transmitted sexually and infects anogenital sites. However, HSV-1 and HSV-2 are both capable of infecting mucosal sites irrespective of their anatomic localization, and can produce clinically indistinguishable lesions. HSV infection causes various forms of disease, from lesions on the lips, eyes or genitalia to encephalitis or disseminated disease (Roizman *et al.*, 2007).

Acyclovir is the antiviral drug of choice for treating HSV infections because of its efficacy and moderate toxicity. Acyclovir is a nucleoside analogue that inhibits the viral DNA polymerase and is clinically used by intravenous, oral or topical route (O'Brien and Campoli-Richards, 1989). Due to its short half-life (about 2 h) and incomplete absorption (bioavailability about 15–30%) (Collins and Bauer, 1977), Acyclovir must be taken in its oral dosage form five times daily (up to 1200 mg/day**)** and the interval of intravenous formulations is of 8 h. Moreover, the intravenous dosage of Acyclovir should be administered slowly over 1 h to prevent its precipitation in renal tubules. The development of delivery systems for Acyclovir administration might improve its efficacy, thus decreasing the need for high and repeated doses, and limit its adverse side effects.

In recent years, nanoparticles have gained increasing attention as drug delivery systems for their several advantages as controlled drug release, protection of active molecules from degradation and cell targeting. They have been proposed as carriers of antiviral drugs for increase their therapeutic index. Nanoparticulate-based systems may change the release kinetics of antivirals, increase their bioavailability, improve their efficacy, restrict adverse drug side effects and reduce treatment costs. Moreover, they might permit the delivery of antiviral drugs to specific target sites and viral reservoirs in the body. These features are particularly relevant in viral diseases where high drug

doses are needed and many active molecules showed a low bioavailability (Lembo and Cavalli, 2010).

The preparation method and the process parameters can markedly affect the formation and the sizes of nanoparticles as well as the amount of drug encapsulated. Size, encapsulation efficiency, surface charge of the nanoparticles are important and stringent properties for the therapeutic application influencing the biopharmaceutical behavior and cellular uptake of nanoparticles.

Consequently the selection of the proper method of preparation plays an important role in the development of drug loaded nanoparticles and the choice mainly depend on either the active molecule characteristics or the type of matrix (generally polymers or lipids).

Moreover, to design nanoparticles as drug delivery systems it should be considered, besides the drug properties, the capacity to obtain a sufficient drug encapsulation.

The large number of preparation methods proposed to obtain nanoparticles can be divided in two general groups: top-down and bottom-up approaches (Verma *et al*. 2009).

The top-down processes consist of the reduction of large particles into smaller particles using milling, high pressure homogenization and microfluidization. These techniques involve high energy and generally considerable heat is generated, consequently they are not suitable for thermolabile compounds. The bottom-up approaches require that the drug is dissolved in an organic solvent with excipients and then precipitated. This approach includes: emulsion-solvent evaporation, emulsion-solvent diffusion, solvent displacement, spray-drying and supercritical fluid processes.

These types of processes can also adversely affect nanoparticles because of the possible formation of hydrates or polymorphs and the presence of residues of the solvents involved in the preparation.

The solvent displacement method, also named nanoprecipitation or solvent injection, is a flexible one-step method firstly developed by Fessi (Fessi, *et al.*, 1989).

The method is suitable for different drugs and various types of substances for the matrix formation of nanoparticles (Schubert and Müller-Goymann, 2003; Quintanar-Guerrero *et al.*, 1998) . Moreover, it is fast, reproducible, cost effective and it is useful to produce particles mainly in the

range of 100-500 nm. This technique requires the use of a solvent completely miscible with water, for example acetone or ethanol or mixtures. The drop-wise addition of a polymer dissolved in acetone to water under stirring formed nanoparticles. The molecular mechanism of particle formation involves complex interfacial phenomena as recently reported (Beck-Broichsitter *et al.*, 2010; Vitale and Kats, 2003; Ganachaud and Kats, 2005).

With this method the particle formation depend on precipitation under condition of spontaneous dispersion or self-assembly of macromolecules (Mora-Huertas *et al.*, 2011).

We choose the solvent displacement method to obtain acyclovir nanoparticles for the simplicity, high reproducibility and absence of toxic solvents or surfactants in the preparation protocol. Moreover it is possible to use preformed polymers and to obtain high encapsulation efficiency of drug in small nanoparticles.

This chapter reports a formulation example of this method of preparation which use a peculiar polymer matrix as a β-CD PACM conjugate. Previously, researches showed that this conjugate present some advantages as stability, biocompatibility, high acyclovir complexation capacity (Bencini *et al.*, 2008). The β-CD-PACM conjugates and water are highly miscible in all proportions and the resultant apparent solutions are stable and not as viscous. Based on these results we used a preformed acyclovir β-CD PACM conjugates to produce the acyclovir loaded nanoparticles (R. Cavalli *et al.*, 2009).

The chapter is divided into three sections. Section one describes the preparation and characterization of β-CDPACM nanoparticles for the delivery of Acyclovir and the investigation of their cell uptake. Section two describes some of the assays that may be used to assess the biocompatibility of the nanoparticulate formulation. Section three provides a description of the methods suitable to assess the antiviral potency of acyclovir-loaded nanoparticles. Overall, the chapter provides a general experimental procedure and methods that should be used to develop nanoparticles aiming at enhancing the therapeutic activity of acyclovir.

**1. Preparation of Acyclovir-loaded nanoparticles and fluorescent nanoparticles**

To prepare nanoparticles a two step procedure is used consisting of the acyclovir of fluorescent marker inclusion in β-cyclodextrin cavity and then nanoparticle formation

*1.1 Preparation and characterization of β-CD-PACM inclusion complexes*

The β-CD-PACM conjugate used is obtained as previously described (Bencini *et al.*, 2008). Briefly, 6-deoxy-6-mercapto-β-ciclodextrin (β-CD-SH) is prepared and used as chain-transfer agent in the radical polymerization of 4-acryloylmorpholine (Sigma, Aldrich, distilled before use, bp 158°C at 50 mmHg). The β-CD-PACM w/w ratio was 32.7 and the overall number-average (Mn) and weight average (Mw) molecular weight values were 7860 and 13500, respectively. Fig.1 reports the schematic structure of the conjugate

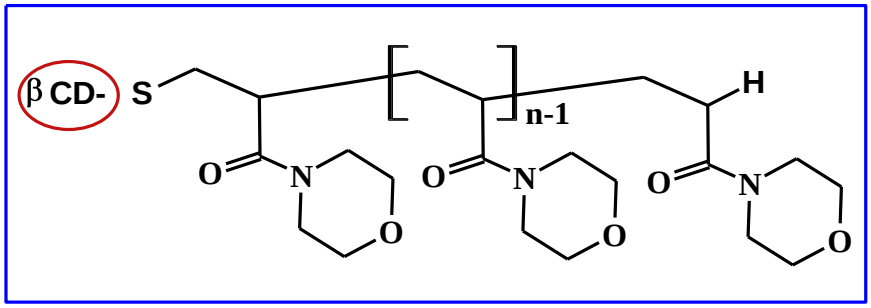


Fig. 1:Schematic structure of β-CD-PACM conjugate

The inclusion complex between Acyclovir (Sigma-Aldrich St. Louis, USA) and β-CD-PACM conjugate is prepared by adding 10 mg of drug to 3 ml of an aqueous solution of the polymer conjugate (10 mg/ml) in a screw-capped tube; the mixture is left to equilibrate for 3 days at room temperature under moderate magnetic stirring and then centrifuged at 5000 rpm for 10 min (Allegra 64 R centrifuge, Beckman, USA). The supernatant is separated and freeze-dried using a Modulyo freeze-drier ( Edwards, UK) to obtain the acyclovir complex in powder form. The solid complex is

used after characterization, for the preparation of the nanoparticles. The Acyclovir β-CD-PACM complex is characterized by Differential Scanning Calorimetry (DSC) and Fourier Transform Infrared (FTIR) spectroscopy.

Thermal analysis is carried out using a DSC/7 differential scanning calorimeter (Perkin-Elmer, CT, USA) equipped with a TAC 7 /DX instrument controller and the Pyris program. The instrument is calibrated with indium for melting point and heat of fusion before the analyses. A heating rate of 10°C/min is employed in the 25-300°C temperature range. Standard aluminum sample pans (Perkin-Elmer, CT, USA) are used and about 3 mg of acyclovir complex are weighted; an empty pan was used as reference standard.. Analyses are carried out under nitrogen purge; triple runs were done on each sample.

For FTIR analysis, KBr pellets (1 cm diameter) are obtained with the complex and a System 2000 instrument (Perkin Elmer) is used to record the spectra in the range between 4000 to 400 $cm^{-1}$.

The amount of Acyclovir complexed is determined by the HPLC method described below, after dilution in a flask of a weighed amount (2 mg) of the complex with a water:acetonitrile (Carlo Erba, Milan, Italy) solution (50:50 v/v).

The quantitative determination of Acyclovir is achieved by HPLC analysis using a Perkin Elmer instrument (L2 Binary Pump, Perkin Elmer) with a Uv-vis spectrophotometer detector (LC 95, Perkin Elmer, USA) with an external standard method. A reverse-phase hypersil ODS column (25 cm x 4.6 mm Varian, USA) is used with a mobile phase consisting of acetonitrile (Carlo Erba, Milan, Italy):20 mM ammonium acetate buffer (Sigma-Aldrich) pH=3.5 (12:88, v/v) at a flow rate of 1 ml/min. The detector wavelength is set at 250 nm. The calibration curve is linear in the range 0.5-15 μg/ml.

The inclusion complex between coumarin 6, chosen as fluorescent marker, (ACROS, Geel, Belgium) and CD-PACM is prepared by adding the fluorescent molecule (2 mg) and 30 mg of the polymer conjugate in 3 ml of distilled water; the suspension is left to equilibrate for 5 days in the

dark, at room temperature and then centrifuged. The supernatant is separated and freeze-dried with a Modulyo freeze-drier (Edwards, UK) to obtain the complex in powder form.

The coumarin 6 β-CD-PACM complex is characterized by Differential Scanning Calorimetry (DSC) using the method described above and by fluorescent spectroscopy ($\lambda_{ex}$ = 450 nm and $\lambda_{em}$= 490 nm) using a RF-551 Shimadzu instrument (Shimadzu, Japan).

*1.2 Preparation of nanoparticles*

The solvent displacement technique is purposely tuned to obtain the β-CD-PACM nanoparticles. An acetone solution containing 13 % v/v of water is selected to disperse the acyclovir PACM complex. Two types of polymer nanoparticles are prepared: blank and Acyclovir-loaded, using the β–CD conjugate as such or pre-loaded as a complex with acyclovir. Briefly, 3 ml of a polymer or a complex in acetone solution (10 mg/ml) are added drop-wise using a microsyringe to 12 ml of filtered water (MilliQ, Millipore) under magnetic stirring at 250 rpm. The aqueous nanoparticle dispersions are then stirred for 5 h at room temperature to eliminate the solvent, washed by diaultrafiltration with a TCF2 Amicon system (Millipore, USA), with a ultrafiltration membrane DIAFLO Amicon cut-off 30,000 Da (Millipore, USA) and stored as aqueous suspension at 4 °C.

Some samples of β–CD-PACM nanoparticles are freeze-dried using a Modulyo freeze-drier (Edwards, UK) to obtain the nanoparticles as a powder.

The same method is used to obtain coumarin 6 loaded nanoparticles

*1.3 Characterization of* β–CD *PACM nanoparticles*

*1.3.1 Sizes and size distribution*

The average diameters and polydispersity indices of the nanoparticle colloidal dispersions are determined by photon correlation spectroscopy (PCS) using a 90 Plus instrument (Brookhaven, NY,

USA) at a fixed scattering angle of 90° and a temperature of 25°C. Each reported value is the average of ten measurements of three different nanoparticle batches. The polydispersity index is the size distribution of the nanoparticle population. The particle size is obtained from the Stokes-Einstein equation

$$D = nKT / (3 \pi \eta d)$$

Where d is the diameter of the nanoparticles

D is the translational diffusion coefficient

K is the Boltzman constant

T is the temperature

$\eta$ is the viscosities of the medium

*1.3.2 Morphology*

Transmission Electron Microscopy (TEM) analysis is performed using a Philips CM10 (Eindoven, NL) instrument. One hundred microliters of nanoparticles are dispersed in 3 ml of filtered water and this nanoparticle suspension is sprayed on Formwar-coated copper grid and air-dried before observation.

*1.3.3 Surface charge*

The electrophoretic mobility and zeta potential of the two types of nanoparticles are determined using a 90 Plus instrument (Brookhaven, NY, USA). For zeta potential determination, samples of the three formulations are diluted with 0.1 mM KCl (Sigma-Aldrich, St.Louis, USA) and placed in the electrophoretic cell, where an electric field of about 15 V/cm is applied. Each sample is

analyzed at least in triplicate. The electrophoretic mobility measured is converted into zeta potential using the Smoluchowsky equation.

*1.3.4 Drug loading and encapsulation efficiency*

The amount of Acyclovir in the nanoparticles is determined by the HPLC method described above. A weighed amount of freeze-dried nanoparticles is dissolved in methanol, diluted and injected into the HPLC system. The loading and encapsulation efficiency are calculated as

$$\% \text{ Encapsulation efficiency (EE)} = \frac{\text{actual amount of drug loaded in nanoparticles}}{\text{theory amount of drug loaded in nanoparticles}} * 100$$

$$\% \text{ Loading amount of the drug} = \frac{\text{amount of drug in nanoparticles}}{\text{amount of drug loaded nanoparticles}} * 100$$

*1.3.5 Determination of the coumarin content in the fluorescent nanoparticles*

The amount of coumarin 6 in the nanoparticles is determined by spectrofluorimetric analysis ) using a RF-551 Shimadzu instrument (Shimadzu, Japan). A weighed amount of freeze-dried nanoparticles is dissolved in methanol and diluted before the sample is measured by fluorescent spectroscopy ($\lambda_{ex}$ = 450 nm and $\lambda_{em}$= 490 nm) using a RF-551 Shimadzu instrument (Shimadzu, Japan).

*1.3.6 In vitro release kinetics*

The *in vitro* release experiments are carried out in phosphate buffer (Carlo Erba, Milan, Italy) at pH = 7.4 at 37 °C using the dialysis bag diffusion technique. About 10 mg of Acyclovir-loaded

nanoparticles is dispersed in 3 ml of 0.05 M phosphate buffer at pH 7.4 and then placed in a dialysis bag using a cellulose dialysis membrane Spectra/Por (Spectrum, CA, USA) with cut-off 3500 Da. The dialysis bag is then placed in 25 ml of phosphate buffer and incubated at 37 °C under stirring. At fixed times within a period of 2 h, buffer samples are collected, replaced with fresh buffer and the concentration of released Acyclovir determined by HPLC analysis in the withdrawn samples using the method previously described. The release of coumarin 6 is carried out at two different pH values to mimic the physiological and intracellular conditions. For the release determination about 2 mg of coumarin 6- loaded nanoparticles are dispersed in 3 ml of 0.05 M phosphate buffer at pH = 7.4 or 0.1 M HEPES buffer (Sigma, Aldrich, USA) pH = 5.0 and then placed in a dialysis bag as previously described. The withdrawn samples collected at fixed time are freeze-dried, reconstituted with methanol and analyzed by fluorimetric spectroscopy) using a RF-551 Shimadzu instrument (Shimadzu, Japan).

*1.4 Sterilization*

For cell experiments sterile formulations are required. For the preparation of sterile nanoparticles all the solution are filtered through a 0.22 μm membrane (Millipore, MA. USA) or autoclaved. The nanoparticles are then obtained in aseptic conditions.

*1.5 Physical stability of β-CD-PACM nanoparticles*

The stability of blank and drug-loaded β–CD-PACM nanoparticle aqueous dispersions is evaluated over time. For this purpose the two types of nanoparticles are maintained at 4 °C. Their average diameter, polydispersity index and zeta potential values are determined after 24, 48 hours and after 1, 3, 6, 12 months. The nanoparticles aqueous dispersions are stable over time without aggregation phenomena

## 2. Biocompatibility assays

Nanoparticles should undergo rigorous processing and characterization as well as safety testing before their application as drug carriers. Safety concerns the evaluation of the biocompatibility and toxic potential of the materials. Toxicity describes a change in form or function of cells while biocompatibility describes the interaction of a material with the host tissues. Once injected in the bloodstream, nanocarriers may interact with erythrocytes and endothelial cells affecting their viability and inducing the production of reactive oxygen species (ROS). Moreover, as any foreign body, they can be taken up from the circulation by phagocytes, including those of the mononuclear phagocytic system. Recognition and elimination of nanoparticles may occur through the absorption of specific proteins, e.g. the C3 protein of the complement system (Sahu *et al.,* 2001). Upon binding, the C3 protein will change conformation, expose a reactive site, and release a signaling molecule that triggers the chain of biochemical events called the complement activation cascade. This will lead to the elimination of the particles by the macrophages, thus preventing the distribution of nanoparticles to other tissues and anatomic districts (Vonarbourg *et al.,* 2006). Therefore, in order to develop safe nanocarriers for medical application and to optimize the pharmacokinetic properties of the agent, it is of paramount importance to select those which are devoid of cytotoxic and complement activation effects and do not induce ROS production. If nanoparticles are designed for topical administration, human cell-derived, organotypic in vitro 3-D tissue equivalents can be used to assess cytotoxicity and induction of proinflammatory cytokines.

### *2.1 Cell viability assay*

First of all, it is important to determine the cytotoxic potential of the formulation on the cell line used in the antiviral assays. This allows to calculate the selectivity index (SI) defined as the ratio of 50% cytotoxic concentration (CC50) to the 50% antiviral concentration (IC50).

The *Vero* cells (ATCC CCL-81) are derived from the kidney of an African green monkey, and are one of the more commonly used mammalian continuous cell lines to propagate HSV and to test anti-HSV compounds.

To test the effect of the Acyclovir complex with β-CD-PACM nanoparticles on the viability of Vero cells, cells are seeded at a density of $6\times10^4$/well in 24-well plates. After 24 h they are incubated with the compounds or left untreated. After 72 h treatment, cell viability is determined by the CellTiter 96 Proliferation Assay Kit (Promega, Madison, WI,USA) according to the manufacturer's instructions. Notably, standard protocol for the MTS test is modified to enable proper evaluation of toxic effect of nanoparticles in cell culture conditions. Preliminary experiments revealed that the optical and chemical properties of nanoparticles might cause errors in the assessment of toxicity using cytotoxicity tests base on optical density measurements. Some of the nanoparticles are able to interfere with the substrate (MTS) increasing amount of formazan formed, even in the absence of living cells. To overcome this limitation, the test must be modified by addition of a second plate where nanoparticles are incubated with MTS in the absence of cells. Optical densities from this plate are subtracted from the optical densities obtained from the cell seeded plate.

The other limitation of the optical density based test is direct influence of nanoparticles on the optical characteristics of the well: to eliminate this limitation the plates are centrifuged and supernatant (free of nanoparticles) was transferred into the second plate (on which the OD measurements were performed). Obtained optical densities are subtracted.

The effect on cell viability of the formulation at different concentrations is expressed as a percentage, by comparing treated cells with cells incubated with culture medium alone. As reported in Fig. , β-CDPACM nanoparticles do not affect viability of Vero cells.

The nanoparticulate formulation should be also tested on endothelial cells.

The Human Umbilical Vein Endothelial Cells (HUVEC) are suitable for cell viability assays. They can be purchased from different sources or, alternatively, can be isolated from normal human

umbilical vein. In this case, aseptically taken umbilical cord, preferably abort 20-30cm length, is clamped and dipped in 70% ethanol for 30s. Umbilical cord (vein) is washed with PBS to remove blood. Collagenase/dispase solution (Collagenase (type I – 1mg/ml (242U/ml)) and dispase (2mg/ml (about 1,4U/ml)) solution in Dulbecco's modified Eagle's medium (DMEM – Gibco) containing antibiotic/antimicotic is run into vessel until it appears at bottom end. Bottom end is clamped and more solution is added to the lumen of vessel. Umbilical cord is incubated at 37°C for 30 minutes (for time to time the cord is rotated). The cell suspension is collected in 50ml centrifuge tube. Lumen of vessel is rinsed with PBS and added to cell suspension. Pooled digest is centrifuged at 200g for 10 minutes. Cells are washed twice and centrifuged. Final pellet is resuspended in culture medium (DMEM:F-12 (1:1) with Glutamax I, antibiotics/antimicotics, 20% FBS, 18U/ml heparin, 10ng/ml EGF) and seeded into flasks. Cells are seeded on collagen coated plastic at conc. $10^4$ per well in 96 well plates. After 24 h of adaptation, cells are exposed to the range of concentrations of nanoparticles. Cell viability is determined by the CellTiter 96 Proliferation Assay Kit (Promega, Madison, WI,USA) as reported previously at different times post-exposure. No cytotoxicity is detectable at the tested concentrations.

*2.2 Evaluation of the hemolytic properties*

Two hundred microliters of nanoparticle suspension are incubated at 37°C for 90 min with 1 ml of diluted human blood (1:5 v/v). After incubation, blood containing suspensions are centrifuged at 1000 rpm for 10 min to separate plasma. The amount of haemoglobin released due to haemolysis is measured spectrophotometrically at 543 nm using a Beckman DM spectrophotometer. The haemolytic activity is calculated with reference to blank and complete haemolyzed samples (induced by addition of ammonium sulfate (Carlo Erba, Milan, Italy) 20 % w/v.

*2.3 Complement activation assay*

Human blood for the experiments is obtained from healthy volunteers.

In a second step, plasma is separated by centrifugation (400g, 10minutes) of heparinised (10 U/ml) (S-Monoovette Sarstedt,Germany) freshly-obtained blood: the separated plasma is then centrifuged once again (800 g, 10 minutes), and later aliquoted in 2 ml polypropylene tubes and stored in -70°C. The processing time must never be longer than 1 hour.

For the assessment of complement activation potential of nanoparticles, human serum is exposed to the tested nanoparticle at a concentration of 1mg/ml in 2ml polypropylene vials for 60 minutes in a standard $CO_2$ incubator (5% $CO_2$, 37°C); the sample is tested in triplicate. After exposure, sample is cooled on ice and mixed with the cocktail of enzyme inhibitors (Sample Stabilizing Solution) to prevent future activation.

Finally, the sample is stored at -70°C for future analysis. Generation of the complement activation products is measured using three different ELISA systems (iC3b, iC4b, Bb, SC5-9; QUIDEL® San,Diego, CA, USA), following the manufacturer's instructions. As a indicator of complement activation iC3b fragment release is chosen.

Briefly, 100µl of iC3b Specimen Diluent (blank), Standards, Controls, and diluted Specimens are pipetted into into the antibody-coated wells. The plate is incubated for 30 minutes at room temperature, then wells are washed for 5 times with the wash buffer provided in the kit and are added to each well 50µl of iC3b Conjugate antibody. The plate is incubated for 30 minutes at room temperature, then the wells are washed five times. Finally, 100 µl of Substrate Solution are added to each well, and the plate is incubated for 15-30 minutes at room temperature. After this incubation time, 50 µl of Stop Solution are added to each well, and absorbance is measured at 405 nm using an ELISA mircoplate reader.

Methodology is based on ISO procedure used for biocompatibility assessment of biomaterials in contact with blood. Obtained results indicate relatively low capacity of tested nanoparticle to activate complement system.

*2.4 Analysis of Intracellular ROS.*

The generation of ROS in treated cells is determined by 2,7-dichlorofluoresceindiacetate (DCFH-DA; Sigma-Aldrich,USA) staining. DCFH-DA is nonfluorescent and can diffuse into the cell through the plasma membrane where it is hydrolyzed to DCFH.

Nonfluorescent DCFH is finally converted to green fluorescent dichlorofluorescein (DCF) upon intracellular oxidation. For this assay, Vero cells are seeded in a 6-well plate ($2X10^5$ cells/ well) and are grown for 24 h.

After this time, cells are treated with different concentrations of nanoparticles, corresponding to ½ $IC_{50}$, $IC_{50}$ and 2 $IC_{50}$, for 3 hours. Then, cells are harvested, and washed twice with PBS.

Finally, the cells are resuspended in 1 ml of MEM with 5 μM DCFH-DA and incubated for 10 minutes at 37°C. Stock (1 mM) solution of DCFH-DA is prepared in ethanol and stored under liquid nitrogen vapor.

Immediately after the incubation, the samples are analyzed for DCF fluorescence in a flow cytometer (FacsCalibur, BD Biosciences, NJ) at an excitation wavelength of 488 nm and emission wavelengths of 530. The fluorescence data are recorded with the CellQuest program (BD Biosciences) for 20000 cells in each sample. Flow cytometric data are analyzed using WinMDI software and the ROS generation is expressed in terms of percentage of cells with DCF (green) fluorescence.

Results indicated that no significant increase of intracellular ROS is detectable at the tested concentrations.

*2.5 Evaluation of skin irritation potential of β-CD-PACM nanoparticles on EpiVaginal Tissue Model*

To evaluate the skin irritation potential of β-CD-PACM nanoparticles on mucous membranes we analyse the toxicity and inflammatory response on a human cell-derived, organotypic in vitro 3-D tissue equivalent. As tissue model, is used the EpiVaginal System, purchased from MatTek

Corporation (Ashland, MA, USA), that consists of Human 3-D Vaginal-Ectocervical Tissues, that is cultured to form a multilayered and highly differentiated tissues closely resembling that of epithelial tissue in vivo. On the base of the foreseen use of the formulation, the study can be extended to other human cell-derived, organotypic in vitro 3-D tissue equivalents, as the EpiAirway and EpiDerm Systems (AIR-100-MM and EPI-200, MatTek Corp.), the differentiated models of the human epidermis and the epithelial tissue of the respiratory tract, respectively. According to the supplier's instructions, the cultures are transferred to 6-well plates (containing 0.9 ml of MatTek assay medium per well) – with the apical surface remaining exposed to air – and incubated at 37°C in 5% $CO_2$ overnight. The cytotoxicity of nanoparticles on mucous membranes is evaluated using two complementary systems: the MTT ET-50 Tissue Viability Assay, to study the metabolic activity of living cells and lactate dehydrogenase (LDH) release, to measure the accumulation of dead cells.

The MTT ET-50 Tissue Viability Assay is a colorimetric assay system that measures the reduction of a yellow tetrazolium component (MTT) into an insoluble purple formazan product by the mitochondria of viable cells. For this purpose nanoparticles (100 μM) is applied to the apical surface at the air-tissue interface of quadruplicate tissues for 30 minutes, 1, 4 and 18 hours at 37°C. When exposure of the samples to the compound is complete, any liquid atop the tissue is decanted and inserts are gently rinsed with PBS to remove any residual material. Then tissues are placed in the MTT solution (300 μl) containing 24-well plate and incubated for three hours at 37°C according the MTT kit's protocol (MatTek Corporation). After the incubation period is complete, the inserts are immersed into a extractant solution to lyse the cells and solubilize the colored crystals for two hours at room temperature on an orbital shaker in the dark. Samples are read using an ELISA plate reader at a wavelength of 570 nm. The amount of color produced is directly proportional to the number of viable cells. The percentage of viability was determined at each of the dose concentrations using the following formula: % viability = 100x(OD(sample)/OD(negative control)) where OD is the optical density. As negative control tissues are incubated with ultrapure water. 1.0% Triton X-100 is used as positive control. We demonstrate that nanoparticles are not cytotoxic

at exposure times and the time required to reduce tissue viability to 50% (ET50) is major of 18 hours (data not shown).

A second standard method for quantification of cellular viability is based on the measurement of cytoplasmic enzyme activity released from damaged cells. Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme which is present in most cells and it is released into the cell culture supernatant upon damage of the cytoplasmic membrane. Released LDH in culture medium of treated tissues is measured with the LDH Cytotoxicity Detection Kit (TAKARA Bio Inc.). The assay is a colorimetric assay system that measures the reduction of tetrazolium salt INT to red formazan, catalyzed by the LDH-catalyzed conversion of lactate to pyruvate. Briefly, 100 μl of culture medium of treated cells is removed and trasfered into corresponding wells of an optically clear 96-well flat bottom microtiter plate. Next, 100 μl of reaction mixture is added to each well and the plates are incubated for 30 minute at room temperature in the dark to avoid photobleaching. Absorbance values for the samples are read using an ELISA plate reader at a wavelength of 490 nm. The following three controls have to be performed in each experimental setup in order to calculate percent cytotoxicity: a background control, to measure the LDH activity contained in the assay medium, a low control, to measure the spontaneous LDH release, that is the LDH activity released from the untreated normal cells, and a high control to measure the maximum releasable LDH activity in the cells, that is the maximum LDH release induced by the addition of Triton X-100.

No difference for the cytoplasmic enzyme LDH release is observed between treated and untreated tissues (data not shown).

The inflammatory response is evaluated monitoring the release of the cytokine IL-1α in the culture medium of treated EpiVaginal tissues at different exposure times of 30 minutes, 1, 4, and 18 hours, as reported previously. After incubation, the concentration of IL-1α in culture medium is measured according to the instructions provided by the manufacturer using the enzyme-linked immunoassay (kit IL-1 alpha ELISA (Bender Medsystem). The kit included a microplate coated with a antibody specific for IL-1 alpha. One hundred microliters of diluent and 50 μl of sample medium is added to

each well in the provided plate, followed by 50μl of biotin-conjugate then covered with a plate cover and incubated for 2 h at room temperature. After washing each well, 100 μl of streptavidin-HRP is added to wells, covered and incubated for 1 h at room temperature. The wells are washed to remove unbound conjugate solution, and 100 μl of TMB substrate solution is added to each well. The plate then incubated in the dark for 20 min. Finally, 50 μl of stop solution is added to each well and absorbance values are measured within 30 min at 450 nm. The concentration of IL-1α is calculated by interpolation of a standard calibration curve. Our results indicate that samples exposed to nanoparticles don't exhibit significant differences in levels of inflammatory cytokine (data not shown).

These data demonstrated that β-CD-PACM nanoparticles not exerted toxicity and inflammatory response on a human reconstructed epithelial tissue and therefore they can be classified as "not irritant" compound.

**3. Assesment of the antiviral activity**

*3.1 HSV-1 infection and Treatment of Vero cells*

The effect of Acyclovir and of the Acyclovir-loaded formulation on the production of infectious viruses of HSV-1 is assessed through a yield reduction assay. Vero cells are grown as monolayers in Eagle's minimal essential medium (MEM, Gibco-BRL) supplemented with 10% of heat inactivated fetal bovine serum (FBS, Gibco-BRL) and antibiotics (Zell Shield, Minerva biolabs). The cells are seeded at a density of $6\times10^4$/well in 24-well plates and after 24 hours are infected with HSV at a multiplicity of infection (MOI) of 0.01 pfu/cell and then exposed to the drug or to the nanoparticulate formulation for 72 h. After 1 h adsorption, the virus inoculum is removed and cultures are exposed in duplicate to serial dilutions of the test compounds. Supernatants are pooled as appropriate when a comlete cytopathic effect occurs in the untreated control (norrmally 48-72

hours post-infection) and cell-free virus infectivity titers are determined in duplicate by the plaque assay in Vero cell monolayers.

*3.2 Virus titration by the plaque assay*

Vero cells are seeded at a density of $1\times10^4$/well in 96-well plates and after 24 hours are inoculated with increasing dilutions of virus inoculum prepared in chilled maintenance medium (MEM, with 2% serum). After 1 h adsorption at 37°C, the virus inoculum is removed, and cells are overlaid with 1.2% methylcellulose and incubated for 72 hours at 37°C. Plates are then fixed and colored with 0.1% of crystal violet for 30 minutes and then gently washed with water. The virus titer is estimated as plaque forming units per ml (pfu /ml) by counting the number of plaques at an appropriate dilution. The percent inhibition of virus infectivity is determined by comparing the virus titer in treated wells to the percent in untreated control wells.

The end-points of the virus yield reduction assay are the inhibitory concentrations of drug which reduced virus yield by 50% ($IC_{50}$) and by 99% ($IC_{99}$) versus the untreated virus control. The percent inhibition of virus infectivity is plotted as a function of drug concentration and the $IC_{50}$ and $IC_{99}$ values for inhibition curves are calculated by using the program PRISM 4 (GraphPad Software, San Diego, California, U.S.A.) to fit a variable slope-sigmoidal dose–response curve.

*3.3 Inhibition of HSV-1 infection in Vero cells by the Acyclovir complex with β-CD-PACM nanoparticles*

The virus yield reduction assay is performed to compare the antiviral activity of plain Acyclovir and Acyclovir β-CD-PACM nanoparticles. The assay provides a stringent test, which allows multiple cycles of viral replication to occur before measuring the production of infectious viruses.

The dose–response curves shown in Fig. and the corresponding $IC_{50}$ and $IC_{99}$ values reported in Table  demonstrate that the antiviral potency of the Acyclovir β-CD-PACM nanoparticles against

two HSV-1 isolates is higher than that of free Acyclovir. By contrast, the unloaded carriers exhibit no antiviral activity per se.

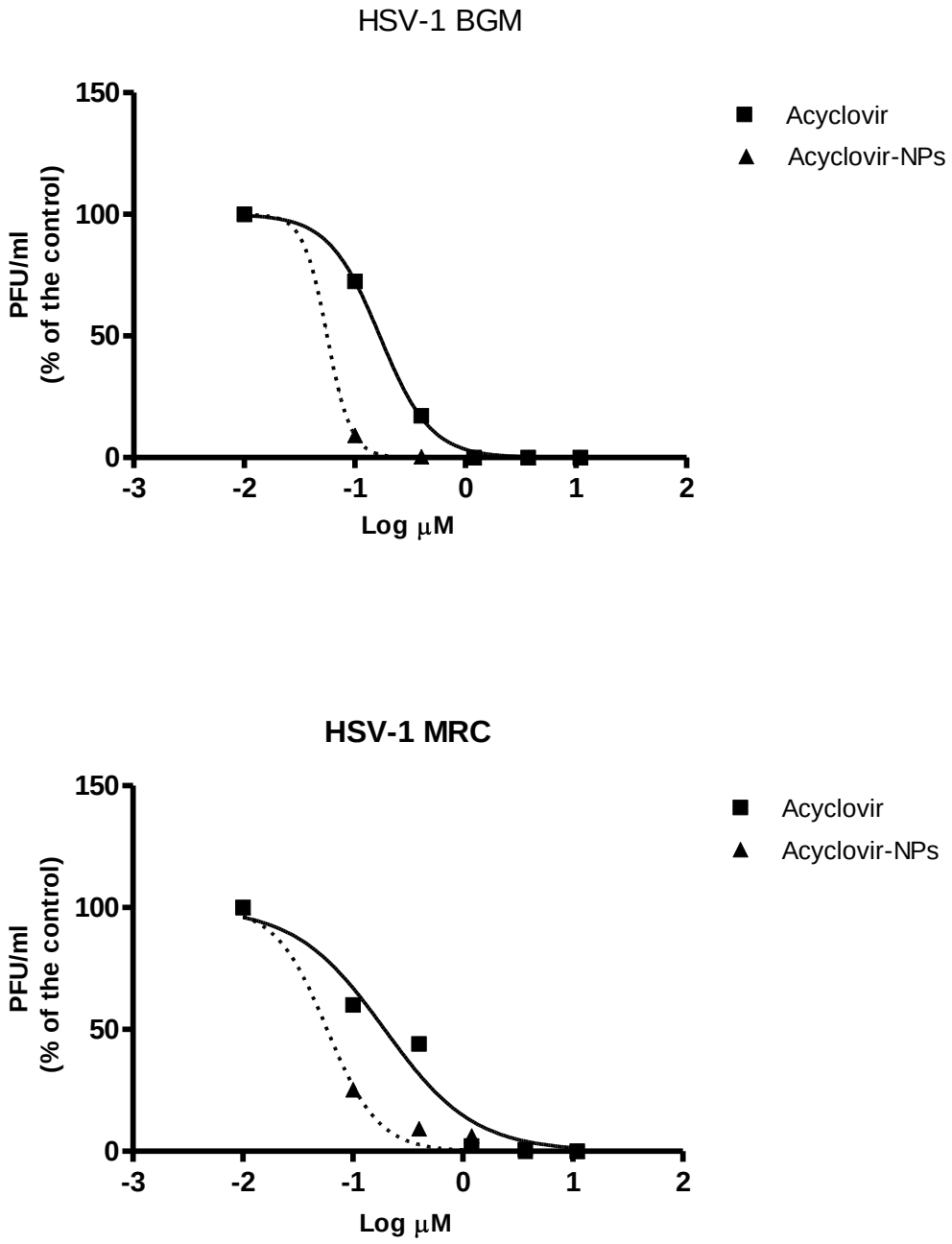


Fig.2 Antiviral activity of Acyclovir and β-CD-PACM nanoparticles on two clinical isolates of HSV-1. Vero cells are infected at a MOI of 0.01 and then exposed for 72 h to different drug concentrations. Supernatants of cell suspension are assayed for their infectivity by standard plaque reduction assay. Values are the means of three separate determinations.

Table 1. Antiviral potency of Acyclovir formulations on Vero cells.

| | HSV-1 BGM | | HSV-1 MRC | |
|---|---|---|---|---|
| | $IC_{50}$[a] | $IC_{99}$ | $IC_{50}$ | $IC_{99}$ |
| Acyclovir | 0.16 | 1.96 | 0.19 | 18.8 |
| Acyclovir-loaded β-CD-PACM nanoparticles | 0.05 | 0.15 | 0.05 | 0.59 |
| β-CD-PACM nanoparticles | >100 | >100 | >100 | >100 |

[a] Values are given as μM

*3.4 Determination of Acyclovir concentration in Vero cells*

The concentration of Acyclovir in Vero cells is investigated as a measure of the intracellular accumulation of the drug. After the incubation with loaded nanoparticles the cells are washed, lysed with a saturated solution of ammonium sulfate and centrifuged at 4 °C for 10 min. The cellular lysate obtained can be frozen until analysis. Just before analysis they are thawed and centrifuged at 5000 rpm for 10 min at 10 °C. The supernatants are diluted with the mobile phase and injected in the system to estimate Acyclovir concentration. The amount of Acyclovir taken up inside the cells is calculated from the standard curve obtained in mobile phase with blank cellular lysate added to varying amounts of drug stock solution. A different reversed-phase HPLC method for the determination of Acyclovir accumulated in cells is developed. A Spherisorb column (250 mm× 4.6 mm, Waters) is used, with water (adjusted to pH= 2.5 with orthophosphoric acid:methanol (Carlo Erba, Milan, Italy) (92:8, v:v) as mobile phase. The detector is set to 252 nm and the flow rate is 1 ml/min. The calibration curve is found to be linear in the range 0.04–10 μg/ml. Uptake enhancement from the formulations is expressed as % uptake versus that of plain Acyclovir.

The intracellular drug concentration is considerably higher when the cells are incubated with Acyclovir-loaded β-CD-PACM nanoparticles than cells incubated with Acyclovir.

*3.5 Evaluation of cellular uptake of coumarin 6 β-CD-PACM inclusion complexes*

The uptake of fluorescently-labeled β-CD-PACM nanoparticles is evaluated by confocal laser scanning microscopy. Exponentially growing Vero cells are plated and cultured overnight in 24-well plates on glass coverslips. The cell monolayers are then incubated with 10 μg/ml of fluorescent labeled nanoparticles for the times indicated and extensively washed with PBS (Carlo Erba) to observe the living cells. The assay is carried out on living unfixed cells to avoid misleading due to the cell fixation protocols. Confocal sections are taken on an inverted Zeiss LSM510 fluorescence microscope. As reported in Fig. , the images indicate that both compounds are internalized soon after exposure to the cells and that they remain within the cell for at least 24 h, with a cytoplasmic distribution. Interestingly, 1 h after treatment the cells incubated with β-CD-PACM nanoparticles exhibit a thicker layer of bright fluorescence in the perinuclear compartment. Fluorescence is not detected in control cells that have not been exposed to the labeled compounds (data not shown). It may be noticed that the perinuclear accumulation of nanoparticles after internalization in cells seems to be a common feature, as reported in several other studies on particulate delivery systems (Desai, *et al*., 1997; Lai *et al*., 2007; Chawla *et al*., 2002; Harus-Frenkel *et al*., 2007; Chavanpatil *et al*., 2007). Based on these results of the cell uptake assay, it is tempting to speculate that the higher antiviral activity of the Acyclovir β-CD-PACM nanoparticle complex is due to the internalization and perinuclear accumulation of the nanoparticles, providing sustained drug delivery in the vicinity of the nucleus, that is the cellular compartment where Acyclovir exerts its antiviral activity.

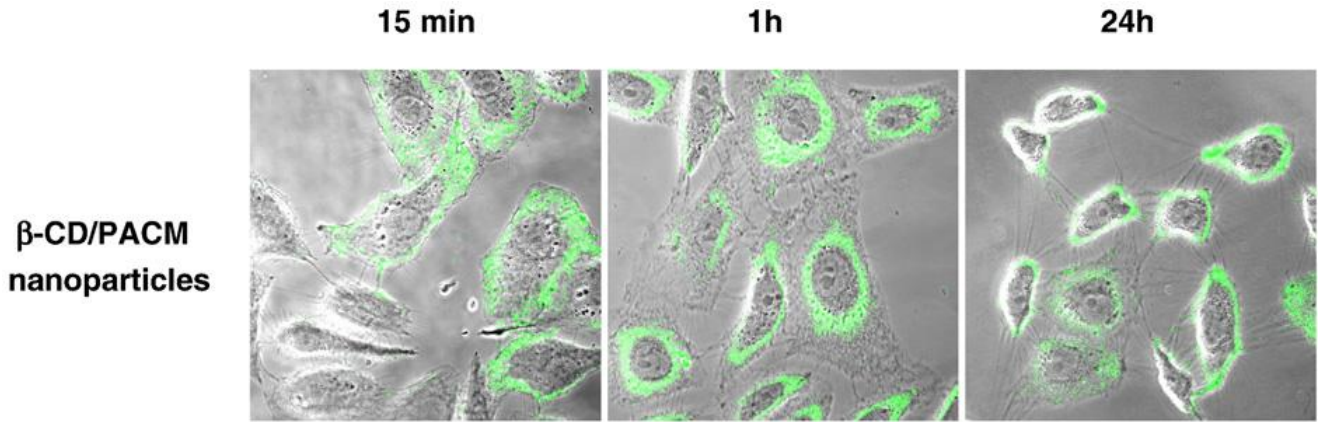


Fig. 3. Cell uptake of β-CD-PACM nanoparticles. Vero cells are incubated with the compound for the times indicated and then analyzed byconfocal laser scanning microscopy without fixation. Merged phase-contrast and immunofluorescence images are shown. β-CD-PACM nanoparticles appear to accumulate in a perinuclear compartment 1 h post-exposure.

## Conclusions

The methods presented in this chapter indicate that acyclovir –loaded nanoparticles with sizes lower than 200 nm and spherical shape are prepared. The antiviral activity of acyclovir in nanoparticles is notably superior compared to the free drug. The nanoparticle formulation may be proposed for the intracellular delivery of antiviral drugs,

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