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Research paper

Increased efficacy of acyclovir-loaded microparticles against herpes simplex virus type 1 in cell culture

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Abstract

Acyclovir is one of the most effective and selective agents against viruses of the herpes group. In order to increase its antiviral activity, acyclovir loaded microparticles, prepared by an O/W solvent evaporation method were developed. Their antiviral activity against herpes simplex virus type 1 (HSV-1) and toxicity were evaluated on Vero cells and then compared with those presented by a drug solution. The 50% inhibitory concentration (IC_{50}) values for acyclovir loaded microspheres determined by plaque reduction assays at 48 and 96 h, were found to be 1.06 ± 0.01 μM and 0.15 ± 0.03 μM, respectively, while the equivalent values obtained for an acyclovir solution were 1.28 ± 0.04 μM at 48 h and 0.27 ± 0.02 μM at 96 h. These results indicate that acyclovir shows a higher antiviral activity, against herpes simplex virus type 1, when this drug was loaded in microparticles rather than as a drug solution, especially after 96 h of incubation. The toxicity of these microparticles was determined by the MTT test at 48 and 96 h. At 48 h only a small toxicity was found (cell viability ranged from 72 to 82%, with the higher concentration tested) and it could not be attributed to the microparticles, since the acyclovir control solution showed similar toxicity values. However, after 96 h a higher toxicity was observed with acyclovir microparticles as well as with the unloaded ones (cell viability located between 60 and 70%). In summary, acyclovir-loaded microparticles have shown to be promising carriers for the effective delivery of acyclovir in the treatment of HSV-1 infections in cells so they can have a potential use in vivo.

Keywords: Acyclovir; PLGA-microparticles; Herpes simplex virus; Antiviral activity; Toxicity

1. Introduction

Acyclovir (ACV), a synthetic analogue of 2′-deoxiguanosine, is one of the most effective and selective agents against viruses of the herpes group [1]. This drug is particularly active against herpes labialis (lesions caused by herpes simplex type 1, HSV-1) and genital herpes (caused by herpes simplex type 2, HSV-2) [2], which remain as common viral infections in humans [3]. Over the past decade, the incidence and severity of infections caused by HSV have increased due to the growth in number of immunocompromized patients, produced by aggressive chemotherapy regiments, expanded organ transplantation and a greater occurrence of human immunodeficiency virus infections.

ACV has shown to be clinically effective in the treatment of HSV-1 cutaneous infections, whose target site is the basal epidermis. In order to increase the effectiveness of this drug, high drug concentrations over a prolonged period of time in the basal epidermis are needed. This fact made interesting the development of particulate delivery systems that could be able to promote the sustained release of the drug in the target site [4]. In this context, PLGA microparticles containing ACV for topical administration have been developed before [5]. In previous works, it has been demonstrated that these microparticles produced a sustained release of the drug into the basal epidermis and subsequently improved the topical therapy [6].

The aim of this paper was to evaluate the antiviral activity of the acyclovir-loaded microparticles against HSV-1 infections in Vero cells. Besides, the cytotoxicity of these microparticles in this type of cells was also studied.

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2. Materials and methods

2.1. Materials

Poly (D,L-lactid-co-glycolid) (PLGA) Resomer® RG 502H 12 kDa has been purchased from Boehringer Ingelheim (Ingelheim, Germany). Polyvinyl alcohol (PVA) was supplied by BDH (Poole, England) and dichloromethane was provided by Prolabo (Fontenay, France). Acyclovir was a gift from Glaxo-Wellcome (Madrid, Spain). Thiazolyl blue (MTT) was purchased from Sigma (Spain). Acetone, methanol and other reagents were obtained from Prolabo (France). Minimum essential medium with Earle's salts (MEM), fetal bovine serum (FBS), penicillin-streptomycin (50 U/ml), hepes buffer 1 M, non-essential amino acids, L-glutamine, Trypsin-EDTA and phosphate buffer saline pH 7.2 (PBS) were obtained from Gibco-BRL (Barcelona, Spain).

2.2. Preparation of acyclovir-loaded microparticles

The microparticles were prepared by a simple emulsion technique as previously described [6]. Briefly, acyclovir was dispersed in a solution of PLGA in dichloromethane. The resulting dispersion was added to a 0.5% PVA solution and homogenized using an ultraturrax® for 1 min. This mixture was stirred at 25 °C until complete solvent evaporation. Microparticles were collected by centrifugation, washed three times with distilled water and freeze-dried for 48 h (Virtis Genesis 12 EL, Gardines, NY, USA). The physico–chemical and morphological characteristics of the microparticles were studied. Unloaded PLGA microparticles (unloaded MP) and acyclovir aqueous solution (ACV) were used as controls. All the samples were dispersed in cell growth medium for the in vitro experiments.

Fluorescently labeled microparticles were prepared in the same way. For this purpose, 4 mg of rhodamine were dispersed in a 16% (w/v) polymer solution in dichloromethane.

2.3. Microparticle characterization

For morphological examination, microparticles were placed on sample holders, 16-mm gold-coated (Emitech K550, UK) and then viewed by using a scanning electron microscope (SEM; scanning digital electron microscope DMS-940A, Zeiss, Germany). Microsphere diameter and size distribution were measured by laser light diffraction (Mastersizer®). The average particle size was expressed as the volume mean diameter in micrometers.

The encapsulation efficiency was determined by using a UV-spectrophotometer (diode array HP 8452 AX, Waldbronn, Germany) at 252 nm. The ACV loaded microspheres were dissolved in 2 ml of dichloromethane, and the drug was extracted twice with 6 ml of 10^{-4} M NaOH. The entrapment efficiency of ACV was calculated as the ratio of actually measured to theoretical (nominal) drug content in microspheres.

The dissolution test was carried out by incubating the microspheres (≈5 mg accurately weighed) into 1.5 ml of phosphate-buffered saline solution (PBS) (pH 7.4) containing 0.02% sodium azide as a bacteriostatic agent. The incubation took place in rotating vials at 37 °C. At predetermined time intervals, namely 1.6 h and 1, 2, 4 and 7 days, the samples were centrifuged at 1700 rpm for 15 min. The ACV concentration in the supernatant was quantified by UV spectrophotometry at 252 nm.

2.4. Cell culture and viruses

Vero cells (African green monkey kidney cell line) were purchased from Vircell (Granada, Spain). This cell line was grown in MEM supplemented with 5% FBS, 1% penicillin–streptomycin, 1% hepes buffer 1 M, 1% non-essential amino acids and 1% L-glutamine. The maintenance medium for Vero cells was as described above but with 2% FBS.

Herpes simplex type 1 (HSV-1) (KOS) was obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA), prepared in aliquots and stored at −80 °C until use. Virus titer was determined by plaque reduction assay in Vero cells and expressed as plaque forming units (pfu) per ml.

2.5. Cell infection

The titer of the virus stocks, determined by plaque reduction assay, was 6.43×10^9 pfu/ml. Monolayers of Vero cells grown in 24-well tissue culture plates, at confluence, were infected by adsorption of HSV-1 (600 pfu), diluted in 100 μl medium supplemented with 2% FBS for 1 h at 37 °C. The inoculum with the non-adsorbed virus was removed and the cells washed with the medium. Finally, the infected cells were treated according to the assay.

2.6. Evaluation of antiviral activity

Acyclovir-microparticles (ACV-MP) were tested for antiviral activity against HSV-1 by plaque reduction assay. This assay was performed according to the standard method described by Hill et al. [7]; 1×10^5 Vero cells were seeded in 24-well plates and incubated at 37 °C and 5% CO_2. When the cells reached 80% of confluence, they were infected with 600 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 acyclovir, ACV-MP or unloaded microparticles. After 48 or 96 h incubation, the medium was removed and the monolayers 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 by using a light microscope. The IC_{50} values were calculated by

regression analysis of the dose–response curves generated from the data.

2.7. Toxicity assays

The cytotoxic effect of the different formulations on Vero cells was tested by using a MTT assay, according to a published method [8]. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) is a yellow water-soluble tetrazolium dye that is reduced by live cells, but not by dead ones, to a purple formazan product that is insoluble in aqueous solutions. Monolayers of Vero cells in 24-well plates were incubated with ACV-MP, unloaded MP or ACV dissolved in complete medium with 2% FBS for periods of 48 and 96 h. Cells were washed with PBS and then MTT was added to the culture wells in order to reach a final concentration of 0.5 mg/ml. The cultures were incubated for 4 h at 37 °C to allow the conversion of MTT to formazan by mitochondrial dehydrogenase. Supernatants were discarded; the formazan crystals were dissolved in an extraction solution (10% sodium dodecyl sulfate in a mixture of dimethyl formamide and water, 1:1, v/v, adjusted to pH 4.7 with acetic acid) overnight at 37 °C. Formazan quantification was performed by measuring the optical density at 570 nm using a 96-well multiscanner autoreader (Labsystems, iEMS Reader MF, Helsinki, Finland) with the extraction solution as a blank.

The cytotoxic effect of each formulation at different concentrations was expressed as a percentage, by comparing treated cells (using the drug solution, ACV-MP and unloaded MP) with cells incubated only with the culture medium.

2.8. Microparticle association with cells

In order to visualize of the association between Vero cells and microparticles, the cells were seeded in Lab-Tek® culture plates (1×10^4 cells/cm^2). When the cells were nearly confluent, the supernatants were discarded and then rodamine-labeled microparticles in maintenance medium were added to each well. After a period of 48 and 96 h, the medium was discarded, the cells monolayers fixed with glutaraldehyde (10%) and the slices observed under fluorescent microscopy (Olympus U-RFLT50, Japan).

2.9. Statistics

All data are presented as arithmetic mean values ± standard error (S.E.). Significant differences were analyzed by using Shapiro-Wilk, *F*-, and Student's *t*-test. A value of $P \leq 0.05$ has been considered significant.

3. Results and discussion

ACV-MP prepared by the simple emulsion technique presented a size of 4.7 ± 0.28 μm. SEM micrographs of the particles showed spherical and well individualized microspheres (photograph not shown) [6]. The encapsulation efficiency was 50 ± 5% (62.5 μg ACV/mg polymer). The release profiles of ACV from PLGA microparticles suggested that the drug release within the first moment was 80% of the actual loading. After the burst, an additional 20% of the drug was released within 7 days.

3.1. Antiviral activity of acyclovir-loaded microparticles

The antiviral activity of ACV-MP against HSV-1 was tested by plaque reduction assay in monolayer cultures of Vero cells at 48 and 96 h. The assays were performed in Vero cells (African Green monkey kidney) since several authors have previously used them as a suitable host for HVS-1 [9–11]. After 1 h of incubation, the virus was removed and then different concentrations of each formulation (ACV-MP, unloaded MP or ACV), dissolved in the medium, were added to the monolayers. The acyclovir carriers exhibited a concentration-dependent response in the plaque reduction assays. Fig. 1a shows the influence of ACV concentration on the HSV-1 replication 48 h after the drug was added even if this was done loaded in microparticles or as a solution. The effective concentration to achieve 50% inhibition of virus replication (IC_{50}) when using ACV-MP and the drug solution was 1.06 ± 0.01 μM and 1.28 ± 0.04 μM, respectively. Consequently, these results showed that, after 48 h, the ACV-MP appeared to be more effective than the drug solution. The plaque reduction assay revealed the absence of activity of unloaded microparticles in infected Vero cells, at concentrations as high as 10 μM (data not shown).

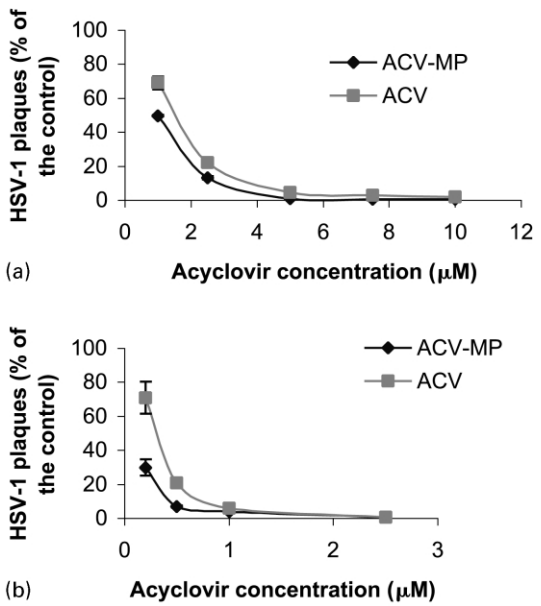

Fig. 1. Effect of different ACV formulations on HSV-1 replication, on Vero cells (a) at 48 h, (b) at 96 h. Each point represents the mean ± S.D. ($n = 3$).

In order to evaluate the effect that the sustained release of the drug from the microparticles has on its antiviral activity, experiments at 96 h were also carried out. When cells were treated for 96 h with the drug solution and ACV-loaded microparticles, the IC_{50} obtained was 0.27 ± 0.02 and 0.15 ± 0.03 μM, respectively (see Fig. 1b). Unloaded microparticles did not present any activity. The improvement of the antiviral activity obtained for both formulations was clearly due to the longer time of contact of the drug with the infected cells. At 96 h, the IC_{50} was reduced 4.7 times for the drug solution and 7.1 for the ACV-MP, compared with the values obtained at 48 h.

In order to clarify if there were some association between Vero cells and microparticles, the cells were incubated with rhodamine-labeled microparticles for 48 and 96 h and then visualized under a fluorescent microscopy. Our results indicated that after both periods of time there were some microparticles associated with the cells, although it was impossible to quantify this association.

This association between the acyclovir loaded microparticles and the cells may explain the higher antiviral activity of the microparticles as these vehicles could be placed on the cells surface and then the drug released there. On the other hand, Ropert et al. [12] suggested that the membrane perturbation caused by the virus might be the key to the uptake of the different colloidal carriers like nanoparticles and liposomes. Infected cells could, thus, constitute a natural target for particulate drug carriers.

3.2. Cellular toxicity

The amount of surviving cells after incubation with an ACV solution, ACV-MP or unloaded MP at different concentrations during 48 or 96 h was estimated by MTT assay. The viability was expressed in percent by comparison with a non-treated control. After 48 h of incubation, the toxicity of all formulations tested was relatively low. Besides, there were no significant differences between them. Fig. 2a shows that at the highest acyclovir concentration tested, 800 μM, the toxicity values were between 18 and 28%. However, when the acyclovir concentration was decreased to 100 μM the toxicity obtained was between 11 and 17%. In all the three formulations, the viability decreased with the drug concentration. These results indicate that the small toxicity found could be attributed to the drug and not to the microparticles, since the ACV control solution showed similar toxicity values. All ACV concentrations assayed in this work were much higher than the active ones (0.01–0.7 μg/ml) [13].

When the MTT assay was carried out after 96 h of incubation, the data obtained suggested that the toxicity of the three formulations have increased with the time of contact of the formulations with the cells (see Fig. 2b). The highest toxicities were observed with ACV-MP and unloaded MP, while ACV was generally less toxic. The viability when the cells were treated with the ACV solution

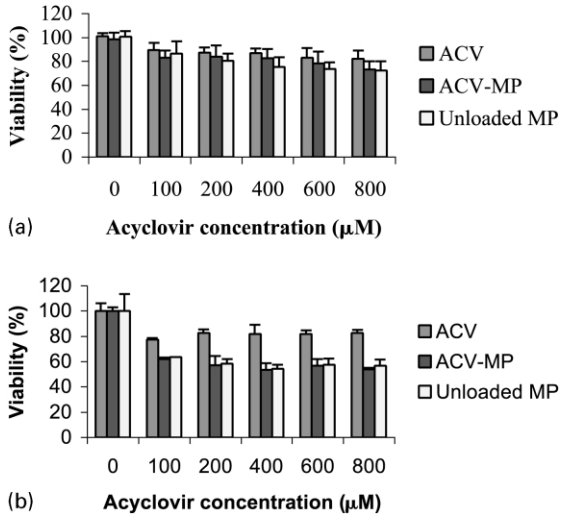

Fig. 2. Effect of different acyclovir formulations on non-infected Vero cells viability, as a function of the drug concentration (a) at 48 h, (b) at 96 h. Each point represents the mean ± S.D. ($n = 3$).

approached 80% for all concentrations of the drug tested. The toxicity values for unload MP and ACV-MP remained practically constant from 100 to 800 μM. These results could be explained by an increase of the local acidity due to the degradation of the polymer, which can lead to cell damage [14]. However, Walter et al. [15] showed that the toxicity of RG 502H microspheres did not increase the proportion of apoptotic or necrotic cells after 7 days of incubation, when they employed cells obtained from human peripheral blood monocytes. In this assay the concentrations used were also much higher than IC_{50} reported.

4. Conclusions

Acyclovir-loaded microparticles are shown to be promising carriers for the topical treatment of HSV-1 infections. The results of the activity studies indicated that ACV-MP was more effective than the drug solution against HSV-1 on Vero cells. This fact is more evident at 96 h, probably due to the sustained release of the drug from microparticles. On the other hand, the toxicity of the ACV-MP and unloaded ones, which was measured using an MTT assay, increases with the time of contact, although it has always exhibited relatively low values. At 48 h, the toxicity induced by these particles was similar than the one presented by the ACV solution, but at 96 h, the toxicity of the carriers increased. This fact can be attributed to an increase of the local acidity due to the degradation of the polymer.

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