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This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/112019> since

Published version:

DOI:10.1016/j.ejpb.2011.10.001

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UNIVERSITÀ DEGLI STUDI DI TORINO

This is an author version of the contribution published on:

Questa è la versione dell'autore dell'opera:

European Journal of Pharmaceutics and Biopharmaceutics, 80(1), 2012,

doi:10.1016/j.ejpb.2011.10.001

The definitive version is available at:

La versione definitiva è disponibile alla URL:

<http://www.sciencedirect.com/science/article/pii/S0939641111002967>

Nonpolymeric nanoassemblies for ocular administration of acyclovir: Pharmacokinetic evaluation in rabbits

Barbara Stella, Silvia Arpicco, Flavio Rocco, Susi Burgalassib, Nadia Nicosia, Silvia Tampucci, Patrizia Chetoni, Luigi Cattel

Abstract

The aim of this study was to increase bioavailability of the antiviral drug acyclovir (ACV) when administered by the ocular route. For this purpose, a new lipophilic derivative of acyclovir was synthesized, both possessing greater lipophilicity and providing the formation of a homogeneous water dispersion with higher amount of ACV than the aqueous solution of the parent drug. This was done by chemically linking acyclovir to the isoprenoid chain of squalene, obtaining 4'-*trisnorsqualenoyl*acyclovir (SQACV), in which squalene is covalently coupled to the 4'-hydroxy group of acyclovir. This new prodrug was then formulated as nonpolymeric nanoassemblies through nanoprecipitation; the resulting particles were characterized in terms of mean diameter, zeta potential, and stability. The pharmacokinetic profile of the prodrug in the tear fluid and in the aqueous humor of rabbits was evaluated and compared to that of the parent drug.

Data showed that SQACV nanoassemblies increased the amount of ACV in the aqueous humor of rabbits compared to free ACV solution. This new amphiphilic prodrug of acyclovir is a very promising tool to increase the ocular bioavailability of the parent drug.

1. Introduction

Topical administration of traditional ophthalmic formulations such as aqueous solutions and ointments is the preferred mode to treat ocular diseases that affect the anterior chamber of the eye, but, unfortunately, the ocular bioavailability of drugs administered in conventional formulations is very limited (2–10%). This is due to the small area available for drug penetration, the presence of absorption barriers, chiefly comprising the lipophilic corneal epithelium, and to the eye's protective mechanisms [1] and [2]. Blinking, baseline, and reflex lachrimation and drainage reduce the contact time of the medication with the corneal surface [3], [4] and [5]. Repeated instillations induce the desired therapeutic effect but cause undesirable side effects, resulting from the systemic absorption through the nasolacrimal duct, estimated to absorb for more than 50% of the instilled dose [6] and [7].

During the last 2–3 decades, hydrogels, mucoadhesive formulations, solid delivery devices (inserts, drug-soaked contact lenses), nano- and microparticulates, and/or chemical approaches to designing bioreversible prodrugs have been investigated, in the attempt to enhance the efficacy of ophthalmic medications, by prolonging the contact time between drug and ocular surface, or by increasing the affinity of the drug for corneal epithelium [8], [9], [10] and [11].

Acyclovir (9-(2-hydroxyethoxymethyl)guanine, ACV), a synthetic analog of 2'-deoxyguanosine, is one of the most effective and selective antiviral drugs. It is primarily used to treat ocular infections caused

by *Herpes simplex virus* (HSV) (e.g. HSV epithelial keratitis), as well as in treating *Varicella-zoster virus* (VZV) infections (e.g. *Herpes zoster ophthalmicus*, HZO) [12], [13], [14], [15] and [16]. Acyclovir is commonly marketed as ophthalmic ointment (3%), because its poor water solubility does not enable the same concentration to be reached in eye drops. Moreover, its low lipophilicity limits acyclovir's passage across the corneal epithelium and, consequently, its bioavailability. Different strategies have been used in order to improve the ocular bioavailability of acyclovir playing on either its water solubility or its lipophilicity to enhance transcorneal passage. In particular, it has been chemically modified to obtain water-soluble prodrugs [17], [18],[19], [20] and [21] or incorporated into several colloidal systems to enhance corneal adhesion; these include nanoparticles [22] and liposomes [23], [24] and [25]. However, it may be difficult to match high concentrations of acyclovir in water with the molecule's increased lipophilicity that could promote drug transcorneal passage but limits its aqueous solubility.

Recently, we conceived a new strategy to increase the therapeutic index of antitumoral and antiviral nucleoside analogs and to administer them more efficiently. This is achieved by coupling them to the acyclic isoprenoid chain of squalene, a precursor in sterol biosynthesis, whose conformation is strongly influenced by solvent polarity [26]. Coupling to the squalene moiety rendered the molecules amphiphilic, and able to spontaneously form spherical nanoassemblies dispersed in an aqueous medium [27], since in polar solvents squalene is tightly coiled [28] and [29]. In particular, this approach was applied to gemcitabine, obtaining 4-(*N*)-*trisnorsqualenoyl*gemcitabine nanoassemblies with a higher anticancer activity than that of the parent drug [27].

In the present study, this experimental approach was applied to acyclovir, in order to obtain 4'-*trisnorsqualenoyl*acyclovir (SQACV). This new acyclovir derivative possesses both greater lipophilicity, due to the squalene tail, and increased water concentration, since it can be formulated as surfactant-free nonpolymeric nanoassemblies, through nanoprecipitation. Moreover, squalene has the added value to be a natural compound, both contained in the outermost oily layer of the tear film [30] and present as a structural component of the corneal epithelium [31].

This paper reports the synthesis of SQACV and the preparation and physicochemical characterization of the resulting nanoassemblies, in terms of mean particle size, polydispersity index, zeta potential and stability. Further, after quantitative analysis of SQACV entrapped within nanoassemblies, the pharmacokinetic behavior of SQACV nanoassemblies was studied in rabbits, measuring both the concentration of SQACV and of its regenerated parent drug (ACV) in tear fluid and aqueous humor.

2. Materials and methods

2.1. Materials and instruments

1,1',2'-*Trisnorsqualenoic acid* was obtained from squalene as described elsewhere [32]. Acyclovir (purity $\geq 99\%$) was purchased from Recordati (Milan, Italy), and dimethylaminopyridine and *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide were from Sigma (Milan, Italy). All other reagents and solvents used were of analytical grade from Carlo Erba Reagenti (Milan, Italy). The ^1H nuclear magnetic resonance (NMR) spectra were recorded on a Bruker 300 Ultrashield instrument (Karlsruhe,

Germany) in CDCl_3 or DMSO at room temperature, with Me_4Si (TMS) as internal standard. Mass spectra were obtained on a Finnigan-MAT TSQ 700 spectrometer (San Jose, CA). The reactions were monitored by thin-layer chromatography (TLC) on F_{254} silica gel pre-coated sheets (Merck, Milan, Italy); after development, the sheets were exposed to iodine vapor. Flash-column chromatography was performed on 230–400 mesh silica gel (Merck, Milan, Italy). All solvents were distilled prior to flash chromatography.

2.2. Synthesis of 4'-trisnorsqualenoylacyclovir (SQACV) (9-[(4E,8E,12E,16E)-4,8,13,17,21-pentamethyl-4,8,12,16,20-docosapentaenoxyethoxymethyl]-2-amino-1,7-dihydro-6H-purin-6-one)

Dimethylaminopyridine (DMAP) (0.120 g, 0.980 mmol) in anhydrous *N,N*-dimethylformamide (DMF) (1.5 ml) was added, under nitrogen atmosphere and at room temperature, to a stirred solution of 1,1',2'-trisnorsqualenoic acid (0.178 g, 0.444 mmol) in anhydrous DMF (1.5 ml). The mixture was then heated to 60 °C. A solution of acyclovir (0.100 g, 0.444 mmol) in anhydrous DMF (2.0 ml) was added dropwise to the stirred reaction mixture at the same temperature. *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide (EDC) (0.188 g, 0.980 mmol) in anhydrous DMF (1.5 ml) was then added. The reaction mixture was stirred for 3 days at 70 °C under nitrogen, and the formation of the desired product was monitored by TLC (ethanol/cyclohexane/chloroform 50:25:25) (Fig. 1). The mixture was then evaporated *in vacuo*; the crude product was dissolved in dichloromethane, washed with HCl 3%, then with saturated brine until neutral pH, dried over anhydrous MgSO_4 , and concentrated under reduced pressure. The crude product was purified by silica gel flash chromatography, after eluting the column alone with dichloromethane/triethylamine, 99:1 and neutralizing with dichloromethane. The product was eluted with dichloromethane/ethanol 95:5. The ester was obtained as a light yellow waxy material; yield: 0.115 g, 42% [33].

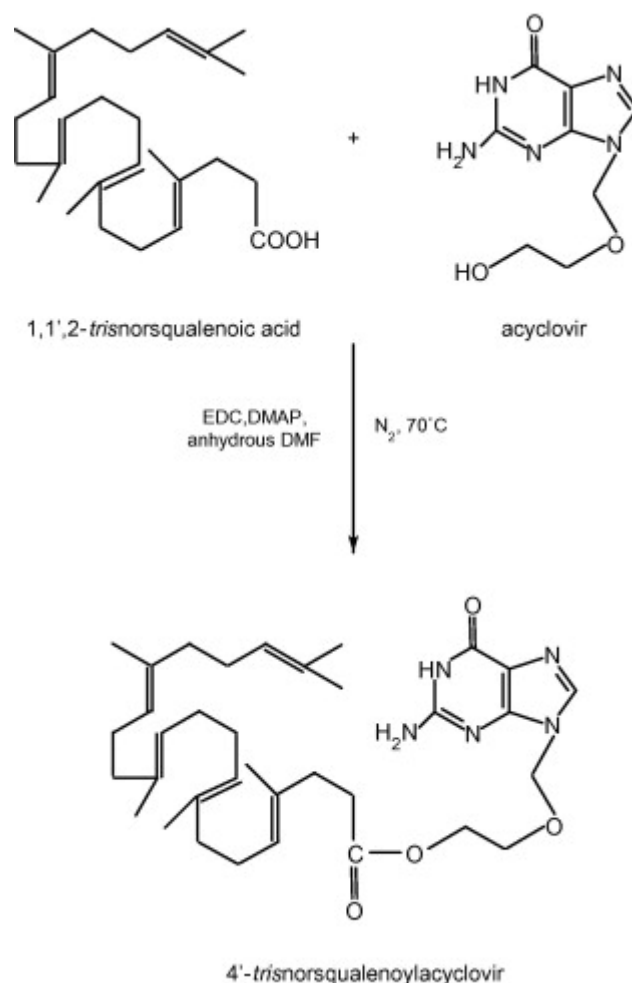


Fig. 1. - Reaction scheme for the synthesis of 4'-trisnorsqualenylacyclovir.

¹H NMR (DMSO, 300 MHz) δ : 10.69 (broad s, 1 H, cycle 1-NHCO), 7.78 (s, 1 H, 8-CH), 6.57 (s, 2 H, NH₂), 5.32 (s, 2 H, 1'-NCH₂O), 5.25–5.03 (m, 5 H, vinylic CH), 4.06 (m, 2 H, 4'-CH₂OCO), 3.63 (m, 2 H, 3'-OCH₂), 2.31 (t, 2 H, OCOCH₂), 2.13 (t, 2 H, OCOCH₂CH₂), 2.10–1.94 (m, 16 H, allylic CH₂), 1.65–1.52 (m, 18 H, allylic CH₃).

Chemical ionization mass spectroscopy (CIMS; isobutane): m/z (%): 608 (100%).

2.3. Preparation of nanoassemblies

SQACV nanoassemblies (SQACV-N) were prepared by the nanoprecipitation technique^[34]. Practically, SQACV was dissolved in acetone (10 mg/ml); this organic solution was then added dropwise under magnetic stirring into MilliQ[®] water (acetone/water 1:2 v/v). Precipitation of the nanoassemblies occurred spontaneously without using any surfactant. After solvent evaporation by Rotavapor[®], an aqueous suspension of pure SQACV-N was obtained. The particle suspensions were then stored at 4 °C.

2.4. Nanoassembly characterization

The mean particle size and polydispersity index of SQACV-N were determined at 20 °C by Quasi-elastic light scattering (QELS) using a nanosizer (Coulter® N4MD, Coulter Electronics, Inc., Hialeah, FL). The selected angle was 90°, and the measurement was taken after dilution of the nanoassembly suspensions in MilliQ® water. Each measure was carried out in triplicate.

The stability of the nanosuspensions was evaluated by measuring the size of the particles after 4 weeks of storage at 4 °C in MilliQ® water. The surface charge of SQACV-N was evaluated by zeta potential measurements after dilution of the suspensions in 10 mM KCl using a Zetasizer (Zeta Potential Analyzer Ver. 2.17, Brookhaven Inst. Corp., Holtsville, NY). The concentration of SQACV in the nanoassembly suspension was checked by HPLC (Merck Hitachi HPLC System, Milan, Italy): an aliquot of particle suspension was evaporated to dryness, dissolved in methanol containing 0.05% v/v trifluoroacetic acid (TFA) before HPLC analysis and then injected into a Symmetry C18 column, 5 µm (Merck, Milan, Italy) equipped with a C18 column guard (Merck, Milan, Italy). The column was eluted with methanol-TFA 0.05% (flow rate 1.0 ml/min). Detection was by UV absorption measurement at 255 nm. Peak heights were recorded and processed on a CBM-10A Shimadzu interface. The drug concentration was calculated from a standard curve. The analytical method was validated, calculating linearity by regression analysis using the least squares method. The assay was linear ($r^2 = 0.9992$) over the tested concentration range (20–1000 ng).

2.5. Preparation of acyclovir formulations for biological studies

An aqueous suspension of SQACV-N was prepared by diluting the pure SQACV dispersion with MilliQ® water to obtain a 0.48% w/w nanosuspension (equivalent to 0.18% w/w ACV) to use for biological studies.

A reference aqueous solution of acyclovir (ACV-S) was prepared by dissolving the drug in phosphate buffer saline (PBS) to obtain an ACV concentration of 0.18% w/w. The osmolality of the preparation was adjusted to physiological values with sodium chloride, and pH was 6.5 [25].

2.6. Biological studies

All experiments were performed in accordance with the ARVO Statement for the Use of Animal in Ophthalmic and Vision Research, following a protocol approved by the Ethical-Scientific Committee of the University of Pisa and under veterinary supervision.

Male New Zealand albino rabbits weighing 2.8–3.5 kg, purchased from Pampaloni Rabbitry (Fauglia, Italy), were used throughout. They were housed in standard cages in a light-controlled room (10 h dark/14 h light cycle) at 19 ± 1 °C and $50 \pm 5\%$ relative humidity. They were fed a standard pellet diet and water *ad libitum*. During the experiments, the rabbits were placed in restraining boxes to which they had been habituated, in a room with dim lighting, and were allowed to move their heads and eyes freely.

Different groups of rabbits, of six animals each, were used to determine (1) ACV retention time in the tear fluid and (2) ACV intraocular penetration studies.

A single dose ($2 \times 50 \mu\text{l}$, at 90 s intervals) [35] of either aqueous dispersion of nanoassemblies containing 0.48% w/w of SQACV or of ACV reference solution (ACV-S) containing 0.18% w/w of ACV was instilled into the lower conjunctival sac of one eye of each rabbit immediately after preparation. The amount of ACV administered was 0.18 mg in either case.

For the precorneal acyclovir retention study, at appropriate time intervals (2, 5, 7, 10, 20, 30 and 50 min) after administration of the formulations, $1.0 \mu\text{l}$ of tear fluid was collected using a microcaps (Drummond Scientific, Broomall, PA), and after appropriate dilution with HPLC mobile phase (1:100 ratio), the samples were submitted to HPLC analysis in the conditions given above.

The aqueous humor acyclovir levels were monitored at 30 and 60 min after instillation of the experimental formulations. Rabbit eyes were anesthetized with $50.0 \mu\text{l}$ of oxybuprocaine hydrochloride (Novesina® 0.5%, Novartis Farma S.p.A., Origgio, Italy), and $50.0 \mu\text{l}$ aqueous humor samples were collected using an insulin syringe (1.0 ml) fitted with a 29G needle (MicroFine, Becton Dickinson, Franklin Lakes, NJ).

The aqueous humor samples were frozen immediately and stored at -18°C . For analysis, thawed samples were mixed with an equal volume of HPLC mobile phase and centrifuged for 5 min at 13,000 rpm (Microcentrifuge 4214, ALC International Srl, Milan, Italy), and $20 \mu\text{l}$ of the samples were submitted to HPLC analysis.

The ocular tolerance and potential irritation of the experimental formulations, SQACV-N and ACV-S, were tested on at least three rabbits by instilling the same dose used in the pharmacokinetic protocol in one eye, while the other eye (reference) was treated with physiological saline. All the eyes were examined by slit-lamp 0.5, 1.0, 3.0 and 12 h after treatment, and the irritation was evaluated according to a scoring scale described in a previous paper [35].

2.7. HPLC analysis of biological samples

Quantitative determination of ACV and SQACV in biological samples was carried out by HPLC. The apparatus consisted of a Shimadzu LC-20AD system with a SIL 10ADvp auto-injector, an UV SPD-10A detector and a computer integrating system. A LiChroCART® 250–4 ($5 \mu\text{m}$; RP-18 endcapped) column was employed. The mobile phase consisted of methanol-TFA 0.05% v/v. The detection wavelength was 255 nm, the flux was 1.0 ml/min, and the retention time was 3.70 and 9.54 min, for ACV and SQACV, respectively.

ACV and SQACV standard curves were obtained by plotting the concentration of known solutions of ACV and SQACV vs the corresponding peak areas of HPLC chromatograms. Both stock solutions of ACV and SQACV and their dilutions were prepared in mobile phase. The analytical method was validated, calculating linearity by regression analysis using the least squares method and precision by injecting six times the standard solutions.

The standard curves were obtained injecting eight standard solutions for ACV and SQACV and were linear ($r^2 = 0.9997\text{--}0.9996$) in the detection range between 0.4345 $\mu\text{g/ml}$ and 5.544 $\mu\text{g/ml}$, and between 1.820 $\mu\text{g/ml}$ and 151.60 $\mu\text{g/ml}$, for ACV and SQACV, respectively [36]. The method was precise in the sample analysis: the coefficients of variation were of 1.33% and 3.32% at 0.80 and 5.0 $\mu\text{g/ml}$ and of 2.45% and 3.53% at 10.0 and 90 $\mu\text{g/ml}$, for six samples of ACV and SQACV, respectively.

2.8. Statistical analysis and pharmacokinetic parameters

Statistical differences between means were assessed by GraphPad Prism software (Prism 5, GraphPad software Inc., San Diego, CA), and the evaluation included calculation of means, standard errors, and group comparisons using the Student's two-tailed unpaired t-test. Differences were considered statistically significant at $p < 0.05$.

The area under the curve values (AUC) of SQACV and ACV concentration in tear fluid were calculated from the first observation (2 min after instillation, $t_{2\text{min}}$) to the end of observation (50 min) from appropriate graphs, applying the linear trapezoidal rule (Prism 5, GraphPad software Inc., San Diego, CA).

The apparent first-order elimination rate constants of SQACV and ACV from tear fluid (K_e) and the corresponding half-lives ($t_{1/2}$) were calculated from the log-linear phase of drug concentration vs time profiles (Prism 5, GraphPad software Inc., San Diego, CA).

3. Results

3.1. Synthesis of 4'-trisinorsqualenoylacyclovir (SQACV)

Pure 4'-trisinorsqualenoylacyclovir was obtained by the esterification of 1,1',2-trisinorsqualenoic acid with acyclovir (Fig. 1). The reaction was highly selective, with no formation of the amidic derivative of acyclovir, as checked by NMR and mass analysis: the signal at about 12 δ of the hypothetical amidic group was absent, while the ^1H NMR spectrum showed the presence of free amino group at 6.57 δ (s, 2 H, NH_2). Analysis of the ^1H NMR spectra of the amide and ester acetyl derivatives of acyclovir reported in the literature confirmed the absence of the amide linkage between the squalene tail and acyclovir [37].

3.2. Preparation and characterization of nanoassemblies

The SQACV prodrug was completely insoluble in water; it spontaneously self-organized into a nanoassembly suspension after dissolution in acetone and addition to water, according to the nanoprecipitation technique.

By varying the drug concentration in the final nanoassembly suspension, preparations of SQACV nanoassemblies with different physicochemical characteristics could be obtained (Table 1). As found by QELS analysis, the SQACV nanoassemblies showed a uniform diameter with a unimodal size

distribution (polydispersity index <0.1), ranging from about 100 to 250 nm, and a negative zeta potential in the range between -33.51 and -41.53 mV (Table 1). After 4 weeks of storage at 4 °C in water, no appreciable size change of nanoassemblies (<5% for all the nanosuspensions) was detected by QELS, and no precipitation or nanoassembly aggregation was observed.

Table 1. - Characteristics of 4'-trisnorsqualenylacyclovir (SQACV) nanoassemblies as a function of their final concentration after nanoprecipitation (means, $n = 3$).

SQACV concentration in final suspension (mg/ml)	Mean particle size (nm \pm S.D.)	Polydispersity index	Zeta potential (mV \pm S.D.)
1	113 \pm 41	0.077	-37.62 \pm 4.50
2	197 \pm 38	0.087	-41.53 \pm 1.98
5	254 \pm 52	0.042	-33.51 \pm 2.99

3.3. Biological studies

The experimental formulations (SQACV-N and ACV-S) were perfectly tolerated: in no case, the relevant test evidenced symptoms of ocular irritation.

The pharmacokinetic behavior of the two formulations (ACV-S and SQACV-N) in tear fluid is illustrated in [Fig. 2](#), as individual profiles of the concentration of ACV and SQACV vs time, resulting from topical administration of ACV-S and SQACV-N formulations, respectively. The total amount of ACV in tear fluid (total-ACV) compared with the concentration of intact-ACV measured after the instillation of ACV-S is also illustrated in [Fig. 3](#). Total-ACV was calculated as sum of ACV produced by biotransformation of the SQACV conjugate (regenerated-ACV) and by transformation of the SQACV experimental data considering the MW of each compound (intact-SQACV).

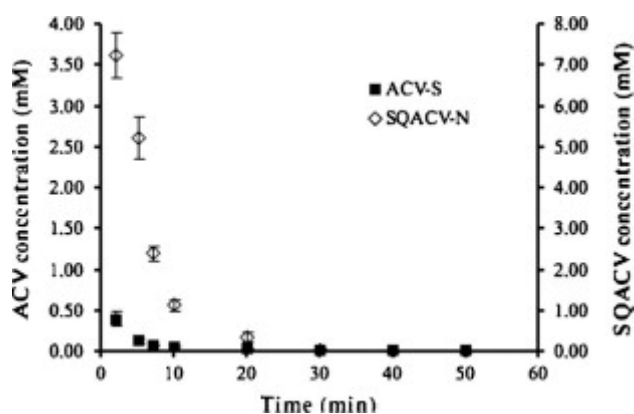


Fig. 2. - Tear fluid concentration vs time profiles of ACV and SQACV upon topical administration of (■) reference ACV aqueous solution (ACV-S) and (◇) SQACV nanoassemblies (SQACV-N) formulations, respectively. (Means \pm S.E., $n = 6$).

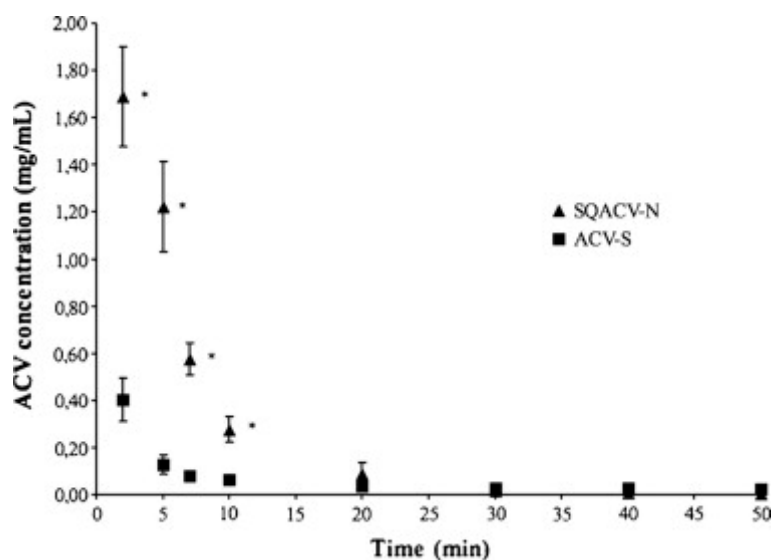


Fig. 3. - Tear fluid concentration vs time profiles of ACV after administration of (▲) SQACV-N (total-ACV) and (■) ACV-S (intact-ACV) formulations. (Means \pm S.E., $n = 6$, *: significantly different from ACV-S ($p < 0.05$)).

Table 2 summarizes the precorneal pharmacokinetic parameters, as apparent first-order elimination rate constants from tear fluid (K_e), relevant half-lives ($t_{1/2}$), concentrations measured at 2 min, AUC values of different species of ACV (regenerated-, intact- and total-ACV) after ocular administration of ACV-S and SQACV-N formulations.

Table 2. - Pharmacokinetic parameters in tear fluid of rabbits (means \pm S.E., $n = 6$).

Formulations	Compound	$C_{2min} (\times 10^2 \text{ mg/ml})$	AUC (mg/ml min ⁻¹)	$K_e (\times 10^2 \text{ min}^{-1})$	$t_{1/2}$ (min)
SQACV-N	Intact-SQACV	434.70 \pm 56.40 [§]	29.23 \pm 5.46	7.46 \pm 0.66 [§]	9.29 \pm 0.97
	Regenerated-ACV	5.99 \pm 1.89 [§]	0.74 \pm 0.16	3.89 \pm 0.41 [§]	17.80 \pm 1.37
	Total-ACV	169.00 \pm 21.00 [§]	11.78 \pm 2.06	6.95 \pm 1.03 [§]	9.97 \pm 2.11
ACV-S	Intact-ACV	40.40 \pm 9.10	2.97 \pm 0.34	10.09 \pm 0.40	6.87 \pm 1.13

§ Significantly different from ACV-S ($p < 0.05$).

As shown in Figures and Tables, higher SQACV tear fluid levels were provided in rabbits by SQACV-N dispersion immediately after their administration (7.245 mM corresponding to 4.347 mg/ml at 2 min) and up to 20 min (0.345 mM corresponding to 0.207 mg/ml). Detectable drug levels were measured up to 50 min after instillation of the nanoassemblies, with a 0.00733 mM concentration of SQACV corresponding to 0.0044 mg/ml. The slow decrease in the SQACV concentration is evidenced by its elimination rate constant and by its half-life, respectively 0.0746 min⁻¹ and 9.29 min. After administration, the SQACV-N dispersion undergoes hydrolysis, producing a reasonably constant amount of regenerated-ACV in the tear fluid, ranging from 0.0599 mg/ml 2 min after instillation to 0.0027 mg/ml at the end of the pharmacokinetic analysis time. The slow elimination rate of regenerated-ACV from tear fluid is evidenced by K_e and $t_{1/2f}$ values of 0.0389 min⁻¹ and 17.80 min,

respectively. The chemical behavior of SQACV determines the presence of significantly higher tear fluid ACV levels with respect to reference ACV-S formulation from 2 min up to 10 min after instillation ($p < 0.05$, see Fig. 3).

Conversely, after instillation of ACV-S solution, the concentration of intact-ACV decreased rapidly in the tear fluid, from 0.80 to 0.13 mM in less than 30 min (corresponding to 0.404 and 0.026 mg/ml, respectively), with a higher elimination rate constant ($K_e = 0.1009 \text{ min}^{-1}$ and $t_{1/2} = 6.87 \text{ min}$), as expected for aqueous solution (ACV-S). The increased residence time of the prodrug in the tear fluid caused a substantial statistical reduction in the elimination of ACV from the tear fluid (K_e : total-ACV = 6.95 min^{-1} vs intact-ACV = 10.09 min^{-1}). The data are clearly indicative of the greater ACV bioavailability produced by the SQACV-N dispersion being more than threefold that of the ACV-S reference solution (AUC = 11.783 and $2.969 \text{ mg/ml min}^{-1}$ for SQACV-N and ACV-S, respectively).

The concentrations of ACV in the aqueous humor of rabbits, 30 and 60 min after topical administration of the formulations under study, are reported in Table 3.

Table 3. - Concentration of ACV in the aqueous humor of rabbits (means \pm S.E., $n = 6$).

Formulations	ACV concentration ($\mu\text{g/ml}$)	
	30 min	60 min
SQACV-N	0.798 \pm 0.206 [§]	0.294 \pm 0.119
ACV-S	0.126 \pm 0.030	0.098 \pm 0.051

[§] Significantly different from ACV-S ($p < 0.05$).

While both intact-SQACV and regenerated-ACV were detected in the tear fluid, after administration of SQACV-N dispersion, only regenerated-ACV was detected in the aqueous humor: its concentration in the anterior chamber was about six- and threefold higher than that measured after instillation of ACV-S solution, at 30 and 60 min, respectively, even if statistically significant differences in the concentration were observed at 30 min exclusively. However, the lowest concentration of ACV detected in the aqueous humor after instillation of the SQACV-N formulation (corresponding to 0.294 $\mu\text{g/ml}$ of the parent drug) was higher than any of those obtained after administration of ACV-S solution.

4. Discussion

This study investigated a combination of two different strategies, in the attempt to develop a promising ocular delivery system, suitable to enhance the therapeutic activity of acyclovir. We selected a prodrug that would increase the lipophilic character of acyclovir, optimizing its permeation through the cornea, and simultaneously the appropriate nanotechnology to provide prolonged

release of acyclovir at the absorption site. Each of these approaches has been widely studied: macromolecular complex, amino acid ester, and dipeptide prodrugs of acyclovir were found to be more stable, more soluble, and capable of improving the bioavailability of ACV after oral and ocular administration [17], [19], [20], [21], [38] and [39], and colloidal carriers have been widely exploited to enhance corneal permeation, control drug release at the absorption site, and obtain a selective target of acyclovir [35], [40] and [41].

The rationale for using a prodrug is the presence of chemical and enzymatic processes that take place in the precorneal area and within the cornea. It is well known that esterases are present in several ocular structures, and their activity is the main factor responsible for the bioreversion of amino acid prodrugs of acyclovir [19], [39] and [42].

Our study employed a lipophilic prodrug of ACV, obtained by conjugation with 1,1',2-trisnorsqualenoic acid, as has been proposed by Sarpietro et al. [33] and [43]. In particular, Sarpietro et al. highlighted the fact that the formation of a lipophilic prodrug gives rise to a stronger interaction with the biomembrane model and is absorbed in larger quantities by the biomembrane model constituted by dimyristoylphosphatidylcholine, than 1,1',2-trisnorsqualenoic acid [33] and [43]. The results reported by Sarpietro et al. using differential scanning calorimetry (DSC) analysis [33] indicated that 1,1',2-trisnorsqualenoic acid and SQACV had a fluidizing effect on the phospholipid bilayer, interacting with the hydrophobic acyl chains, while acyclovir does not enter the phospholipid bilayers [44].

Moreover, squalene's conformation is strongly influenced by solvent polarity, so that in polar solvents it is tightly coiled. This unique property is maintained in squalene-drug conjugates, such as SQACV, so that it is possible to obtain nanoassemblies from these derivatives in water without adding any surfactant. Squalene is also a structural constituent of the corneal epithelium, being a precursor of cholesterol, and in addition, it is a component of the lipid layer of the precorneal tear film, together with wax esters, cholesterol esters, triglycerides, diglycerides, monoglycerides, free fatty acids, free cholesterol, phospholipids, sterol esters, and polar lipids [31].

Herpetic keratitis, defined as the corneal infection caused by *Herpes simplex virus type 1* (HSV-1), may be superficial when it affects only the corneal epithelial cells, or may affect the deeper layers of the cornea, causing a pathological condition known as stromal keratitis. ACV is utilized exclusively in superficial corneal keratitis, as a result of its poor corneal permeability and its limited paracellular diffusion. Enhancement of the residence time of ACV in the tear fluid, and/or of its penetration into the corneal structure to reach the aqueous humor, would thus appear a promising therapeutic goal that could be achieved using the SQACV prodrug.

As demonstrated in this work, the ocular administration of SQACV prodrug increased the bioavailability of ACV in both the tear fluid and in the aqueous humor, as much as four- and sixfold the reference values, respectively. The intact prodrug was found in the lacrimal fluid, while it was not detected in the aqueous humor, thus ruling out passive transcorneal diffusion of the conjugate. Anyway, its lipophilic character associated with its high affinity for the ocular structure could improve the availability of ACV on the corneal surface, as highlighted also from the significant reduction in the elimination rate constant (K_e).

All these considerations partially explain the high bioavailability of ACV after instillation of SQACV nanoassemblies. The longer residence time of SQACV in the precorneal area compared to ACV solution could be due to the technological characteristics of the formulation.

Although several studies have reported data on the increase in ocular bioavailability after administration of positively charged colloidal formulations, due to interaction with the corneal epithelium, the presence of a negative charge, as in SQACV-N (mean zeta potential about -33 mV), does not seem to be a limiting factor [45] and [46]. Furthermore, it should be borne in mind that particulate systems with a size of 100–200 nm can be retained on the corneal surface, and also in the conjunctival sac, for longer times than can an aqueous solution of the drug, avoiding the loss via tear turnover, as demonstrated by the $t_{1/2}$ value calculated for SQACV nanoassemblies ($t_{1/2} = 9.29$ min) [11].

The ACV EC_{50} value for HSV-1 in the aqueous humor is 7.1 μM [20], corresponding to a concentration of 1.6 $\mu\text{g/ml}$. Although the formulation SQACV-N determines an active concentration of ACV in the tear fluid, its concentration in the aqueous humor remains below the EC_{50} value. However, the good ocular tolerability of the nanoassemblies prompted us to hypothesize the possibility of increasing the instilled dose of SQACV-N, and thus the acyclovir level in the aqueous humor, making the formulation useful in the case of stromal keratitis.

5. Conclusions

The linkage between acyclovir and squalene afforded a new amphiphilic prodrug, SQACV, which forms nanoassemblies without surfactants. These nanoassemblies are more advantageous than the free nucleoside analog in solution, since they provide the formation of a homogeneous water dispersion with high amount of ACV and, at the same time, increase its ocular bioavailability. Studies are in progress to further characterize the nanoassemblies (e.g. with electron microscopy, TEM and SEM) and to improve the formulation in order to achieve adequate therapeutic activity.

Acknowledgments

This work was supported by MIUR ex 40% – Progetto Nazionale Tecnologie Farmaceutiche and by the Regional Government (Regione Piemonte).

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