In Vitro Delivery of Novel, Highly Potent Anti-Varicella Zoster Virus Nucleoside Analogues to their Target Site in the Skin

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Purpose. To determine the *in-vitro* dermal delivery of a new class of lipophilic, highly potent and uniquely selective anti-VZV nucleoside analogues in comparison with aciclovir.

Methods. Three test compounds (Cf1698, Cf1743, Cf1712) and aciclovir were formulated into propylene glycol/aqueous cream BP formulations and finite doses applied to full-thickness pig ear skin for 48 hours in vertical Franz-type diffusion cells. Receptor phase samples were taken at specific intervals to determine permeation, and depth profiles were constructed following tape stripping and membrane separation.

Results. All three test compounds reached the target basal epidermis in concentrations suggesting they would be highly efficacious in reducing viral load. Furthermore, the data showed that each of the test compounds would perform in a far superior manner to aciclovir, the current treatment of choice.

Conclusions. The dermatomal site of viral replication during secondary infection—the basal epidermis—was successfully targeted. Topical delivery of these compounds is highly promising as a new first line treatment of VZV infections. By attacking the virus at the first sign of reactivation, it is proposed that the extent of damage caused by the virus would be significantly lowered, thereby limiting the extent and severity of post-herpetic neuralgia.

KEY WORDS: antiviral, topical, herpesvirus, zoster, shingles, PHN.

INTRODUCTION

Varicella Zoster Virus (VZV) is a human alphaherpesvirus, designated human herpesvirus 3, genetically similar to the herpes simplex viruses (1). Primary infection typically occurs in childhood, via the respiratory tract, and leads to the development of chickenpox (varicella) and the establishment of latent virus in the sensory ganglia. Reactivation of the virus, usually occurring years after the primary infection, leads to the development of shingles (zoster) (2) which is often accompanied by pain that can last for months or even years after the initial shingles rash has disappeared. Such prolonged pain, referred to as post-herpetic neuralgia (PHN), is the most common complication of varicella zoster infection, primarily because of its lack of response to traditional analgesic treatments, or even alternative treatments that are frequently relied upon for nerve pain, such as anti-depressants and anti-convulsants (3). The reported level of pain relief from the use of tricyclic antidepressants, one of the most effective treatments available, is as low as 50% (4).

The ideal option would be for an effective system for the prophylaxis of PHN. Antiviral treatment has been used and has shown mixed results: in some cases aciclovir has reduced the duration of pain, while in other cases it has had no reported effect (3). Antivirals have been used to treat PHN because of the perceived pathology behind the condition: after reactivation and transfer to the skin, the virus replicates again (5) and damages peripheral and central nerve fibres (6). Replication of the virus in the affected dermatome causes inflammation that leads to epidermal necrosis, resulting in characteristic blistering (7) and damage to the epidermal nociceptors, which is responsible for the increased incidence of pain (8). By eradicating the virus as it replicates it is proposed that the damage caused to the nerves will be reduced, subsequently lowering the extent of PHN experienced. There are two main problems with this approach. Firstly, the best currently available treatment, aciclovir, is poorly effective, probably as it is not specific to the virus. Secondly, the situation is exacerbated in that aciclovir is a hydrophilic molecule (calculated log of octanol/water partition co-efficient of –1.7, (ClogP v.1.0, Biobyte Corp., 1994)) and consequently is poorly taken up into skin of affected areas from topical preparations, failing to reach the basal epidermis in adequate concentrations (9). It is suggested that a more effective antiviral regime would help reduce the incidence of PHN significantly (10).

A new class of anti-VZV nucleoside analogues has recently been discovered, the most potent of which shows VZV EC_{50} values *in vitro* that are approximately 30,000 times superior to those of aciclovir (11). These compounds were found to be uniquely specific to VZV and entirely non-toxic *in vitro*, at therapeutic concentrations and above. In addition, it was hypothesised that the lipophilic nature of these compounds (ClogP range for entire family of 0.4 to 4.6, (ClogP v.1.0, Biobyte Corp., 1994)) would make some of them suitable candidates for topical delivery and development, in particular, as first-line treatment for shingles. In this report, initial permeation studies were carried out *in vitro* with three members of the group of compounds (Fig. 1), from saturated suspensions in propylene glycol through full-thickness excised pig ear skin as a model for human skin (12,13). This was followed by a study of the dermal penetration of the three compounds *in vitro,* following formulation as creams and application to full-thickness excised pig ear skin. As a comparison, the same penetration studies were also performed with aciclovir formulated into an identical cream.

MATERIALS AND METHODS

Materials

The active compounds (Cf1698, Cf1743 and Cf1712,) were synthesised according to published methods (11). Aciclovir was a gift from Cox Pharmaceuticals (Barnstaple, UK). Acetonitrile and cream excipients (emulsifying ointment, phenoxyethanol, propylene glycol) were obtained from Fisher (Loughborough, UK). Pig ears were obtained from a local abattoir prior to steam cleaning and immediately frozen. Tape stripping was performed using 14 mm D-Squame skin sampling discs (CuDerm Corp., Dallas, Texas, USA).

Preparation of Saturated Suspensions in Propylene Glycol

1% (w/w) suspensions of each compound in propylene glycol were made by weighing out 100 mg of compound and

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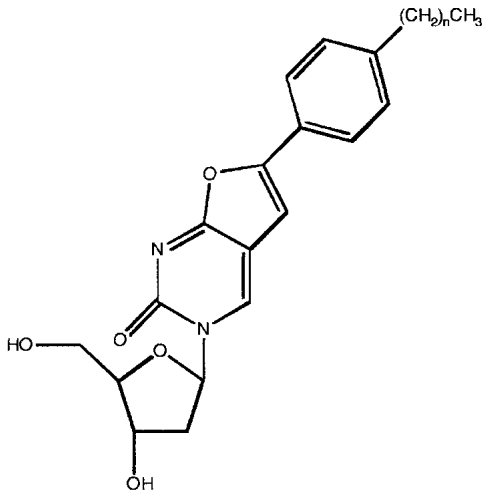

Compound	n	Mol Wt	EC_{50} (μM) (VZV)		CC_{50} (μM)	ClogP
			OKA	YS		
Cf1698	2	370.40	0.01	0.008	188	1.9
Cf1743	4	398.45	0.0003	0.0001	>200	3.0
Cf1712	6	426.51	0.005	0.003	18	4.0
(Aciclovir	-	225.20	1.9	2.1	>200	-1.7)

Fig. 1. Three compounds from the family selected for their activity profiles against VZV (OKA and YS strains) and ClogP values, compared with aciclovir (data adapted from McGuigan C et al, 2000).

making it up to 109 with propylene glycol. Each suspension was stirred rapidly with magnetic followers at 32°C for 24 hours.

Formulation of Creams

The base for the formulation was Aqueous Cream BP, made according to the guidelines in the British Pharmacopoeia. The formulation for 100 g of Aqueous Cream BP was as follows:

- Emulsifying Ointment BP 30 g
- Phenoxyethanol 1 g
- Freshly boiled and cooled purified water to 100 g

The phenoxyethanol was dissolved in the water with gentle heating, while the emulsifying ointment was heated to approximately the same temperature. Once the emulsifying ointment has melted and the phenoxy ethanol had fully dissolved, the aqueous solution was added to the emulsifying ointment and the mixture stirred until cool to form the cream. Emulsifying Ointment BP consists of emulsifying wax (30%), white soft paraffin (50%), and liquid paraffin (20%), and acts as a hydrophobic vehicle, structural matrix former and emulsifying agent. Phenoxyethanol has bactericidal properties and so was present as a preservative.

Owing to their low solubility in a range of excipients, the three Cf compounds could not be incorporated into the cream formulation by dissolution in one of its components or phases. Therefore each test compound was suspended and sonicated in propylene glycol, and incorporated into Aqueous Cream BP during formulation. Rheological studies were performed prior to this to analyse the stability of the cream using varying quantities of propylene glycol in place of water, and a maximum percentage of 30% propylene glycol was found to be stable (rheological data not shown). During formulation of the Aqueous Cream BP, 30% water was omitted, and a 1% (w/w) suspension of Cfl698 in propylene glycol, identical to that used in the previous permeation experiment, was added to the mixture in its place. The aqueous solution of phenoxyethanol and the propylene glycol suspension were both warmed separately, and added simultaneously to the melted emulsifying ointment. The mixture was then stirred until cooled to give the final active cream. This was repeated for Cf1743 and Cfl712, giving three creams each at 0.3% (w/w) concentration of active compound, and then the same was done with aciclovir to give a comparative 0.3% cream.

In vitro Skin Permeation

Full thickness skin was excised from the outside of four pig ears by blunt dissection and cut into 2 cm^2 sections. The skin was placed in all-glass Franz-type diffusion cells, sealed around the edges with silicone grease, and containing degassed water as the receptor phase, as a simple representation of *in vivo* conditions. 0.5 ml of the 1% (w/w) suspension of Cf compound in propylene glycol, prepared as described above, was added to each donor compartment (n = 12 per compound). The average diffusional surface area of the cells (±SD) was 1.11 ± 0.03 cm^2. The receptor phases were stirred using magnetic followers, set up in a water bath set at 37°C to give a skin surface temperature of 32°C. 200 μl samples were taken from the receptor phase after 1, 2, 4, 6, 12, 24, 36, 48, 60 and 72 h, each time being replaced with fresh receptor phase. After 72 h the experiment was stopped, and the receptor phase samples were ready for HPLC analysis.

In vitro Skin Penetration

The Franz cells were set up in the same way as in the permeation experiment, with full-thickness pig ear skin and degassed water as the receptor phase. Creams were applied to the stratum corneum side of the skin using a glass rod and a microspatula (n = 12 per compound), allowing an average (±SD) of 3.78 ± 0.91 mg of cream cm^{-2} (11.34 ± 2.73 μg of compound). The average diffusional surface area of the cells (±SD) was 1.11 ± 0.03 cm^2. The receptor phases were stirred using magnetic followers, set up in a water bath set at 37°C to give a skin surface temperature of 32°C. 200 μl samples were taken from the receptor phase after 1, 2, 4, 6, 12, 24, 36 and 48 h, each time being replaced with fresh receptor phase. After 48 h, the skin was recovered from the diffusion cells and the surface swabbed with a cotton bud dampened with water to remove any remaining excess cream residue.

Localisation of the penetrants within the skin tissue was studied using the technique of tape stripping, in which successive strata of tissue are removed using adhesive discs and analysed to provide comparative depth profiles (14,15). A total of 15 tape strips were applied and removed in succession from the surface and were grouped as 1–3, 4–6, 7–10 and 11–15. The remaining epidermal tissue was then excised from the dermis using a scalpel and forceps. The grease from the diffusion cell was also removed using a cotton bud to check for uptake of permeant. The samples were placed in 8 ml vials and either 3 ml acetonitrile (for Cf1698, Cf1743 and Cf1712) or 3 ml water (for aciclovir) was added to each to dissolve the

permeant (except for the grease samples, to which 3 ml THF was added). The vials were left to rock for 24 hours, after which the solutions were recovered, centrifuged, and transferred to HPLC vials ready for analysis. The original samples were extracted on a second occasion using the same protocol to ensure maximal recovery of penetrant.

Analytical Method

Samples were analysed using an Agilent 1100 automated HPLC system, comprised of a G1322A degasser, G1311A Quaternary Pump, G1313A ALS autosampler, G1321A FLD fluorescence detector and G1314A VWD UV detector, fitted with a Sphereclone® 5 μ ODS 4.6 × 250 mm column (Phenomenex, Macclesfield, UK). For the three test compounds, the mobile phase consisted of acetonitrile and water in ratios of 50:50 (Cf1698), 60:40 (Cf1743) and 70:30 (Cf1712), delivered at a rate of 1.0 ml min^{-1}. Samples (20 μl aliquots) were injected and the compounds were detected with the fluorescence detector using an excitation λ of 347 nm and emission λ of 425 nm. The retention times were 5.4 min (Cf1698), 5.7 min (Cf1743) and 6.9 min (Cf1712). For aciclovir, a gradient mobile phase system was used, with eluent A consisting of 5 mM NH_4OAc in $MeOH/H_2O$ (5:95), and eluent B consisting of 5 mM NH_4OAc in MeOH. The ratio of A:B was 90:10 at t = 0, 55:45 at t = 4, 20:80 at t = 6, and 90:10 at t = 10, with a stop time of 15 min (16). Detection was performed with the UV detector at λ of 283 nm. Analysis was performed using Chemstation software. Calibration curves for the test compounds were linear over the range of 0.05–1 μg ml^{-1} with a limit of detection of 0.2 ng ml^{-1}, and for aciclovir the calibration was linear over 0.05–1 mg ml^{-1}, with a limit of detection of 0.2 μg ml^{-1}. The R^2 value for each calibration was in the range of 0.9968 to 0.9996.

RESULTS AND DISCUSSION

Permeation from Saturated Solutions

Of the three test compounds, only the most hydrophilic compound, Cf1698, was detected in the receptor phase (Fig. 2). Although both Cf1743 and Cf1712 only differ from Cf1698 by having 2 and 4 extra carbons respectively in the alkyl chain, they appeared not to fully permeate the skin despite exhibiting detectable levels of solubility in water (approximate values: Cf1698 10.5 μg ml^{-1}, Cf1743 3 μg ml^{-1}, Cf1712 0.5 μg ml^{-1}). The rate of flux increased continuously throughout the experiment, even after 48 h, and so the steady-state portion of the graph was difficult to determine. However, the relatively linear portion between the 48 hand 72 h provides a steady-state flux of 12.1 ng cm^{-2} h^{-1}, with a lag time of 36.5 h (as determined by extrapolation of the linear portion of the cumulative permeation profile to the X-axis). Whether this was indeed the true steady-state, or whether it is a factor of declining barrier function after two to three days under the experimental conditions used is unclear. Regardless of the steady-state flux of Cf1698, it was the only compound to permeate through to the receptor phase, and therefore showed significantly different behaviour from Cf1743 and Cf1712, which remained resident within the skin.

Permeation and Penetration from 0.3% Creams

Of the three test compounds, once again only the most hydrophilic (Cf1698) was detectable in the receptor phase. The steady state flux was 4.89 ng cm^{-2} h^{-1}, with a lag time of 18 h. This was approximately 40% of the maximum flux observed from a saturated propylene glycol receptor phase, which extrapolated to a lag time of 36.5 h. Cf1743 and Cf1712 failed to reach the receptor phase to any detectable level again. Aciclovir permeated into the receptor phase at a greater rate than Cf1698, with a steady state flux of 500 ng cm^{-2} h^{-1}, and a lag time of 3.5 h. This might be considered as unexpected due to the hydrophilic nature of this compound, although the presence of 30% propylene glycol in the formulation is likely to be responsible for enhancing the penetration of aciclovir through its main obstacle, the lipophilic stratum corneum. It is also possible that the aciclovir traveled across the membrane via a different route, for example through a porous pathway, as has been proposed for polar molecules in previous investigations (15,17–20).

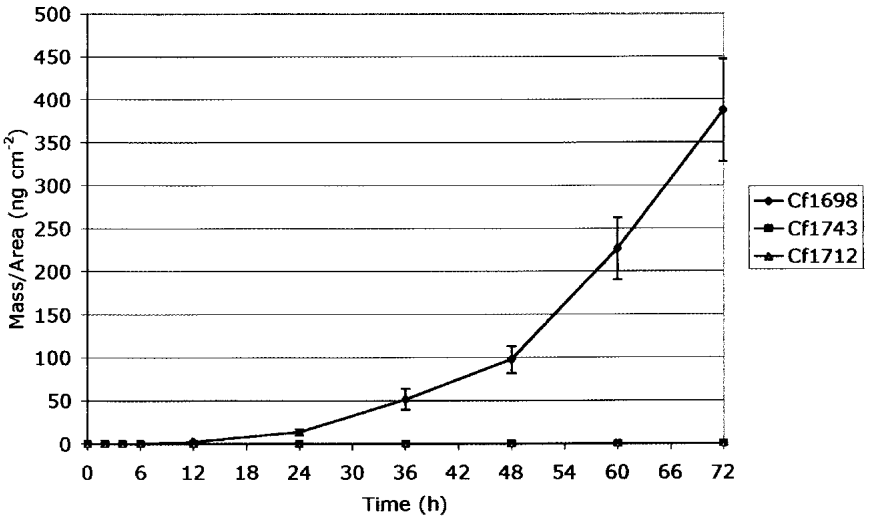

Fig. 2. Average receptor phase concentrations after permeation from saturated suspensions in propylene glycol (±SEM, n = 12).

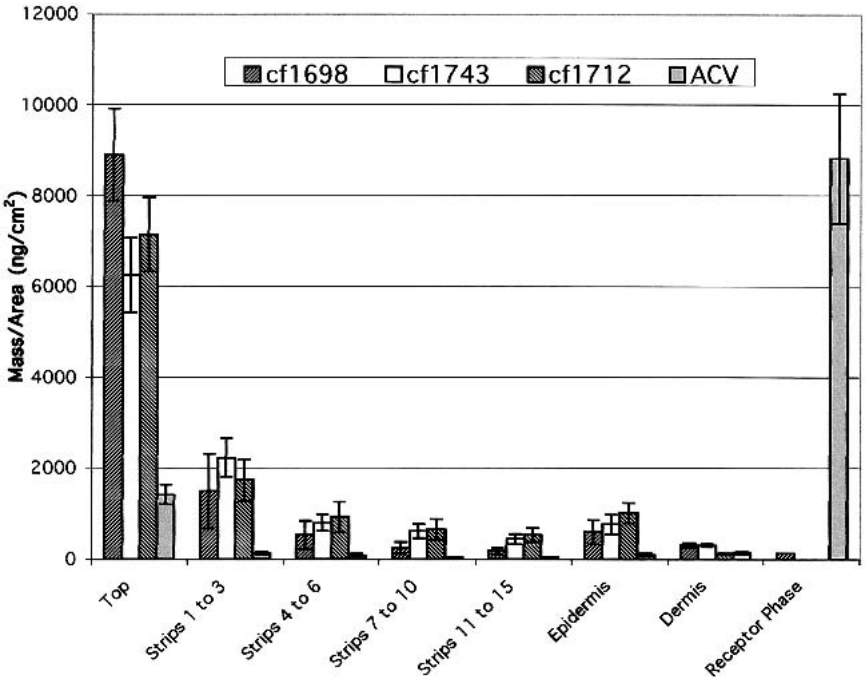

Fig. 3. Average tape stripping and extraction results after 48 hrs, from stratum corneum surface to dermis, and final receptor phase, from cream formulations (±SEM, n = 12).

Figures 3 and 4 show the localisation of the penetrants in the pig ear skin following tape stripping and skin extraction. As can be seen from these results all three of the test compounds, as well as aciclovir, reached the epidermis and dermis. Although slightly variant distributions are apparent, two-tailed Student's t testing demonstrated no significant difference between any of the test compound results at $p = 0.05$ for the epidermis, while in the dermis, only Cf1712 was significantly lower in concentration than Cf1698 and Cf1743 at $p = 0.05$. Aciclovir was significantly lower in concentration than all three test compounds in the stratum corneum and epidermis, but is only significantly lower than Cf1698 and Cf1743 at the dermis, and was not significantly different from Cf1712 at $p = 0.05$.

The distribution of the three test compounds, although generally not varying by a very significant degree, still exhibited some trends that can be explained by the relative alkyl chain length and lipophilicity of each compound. Although

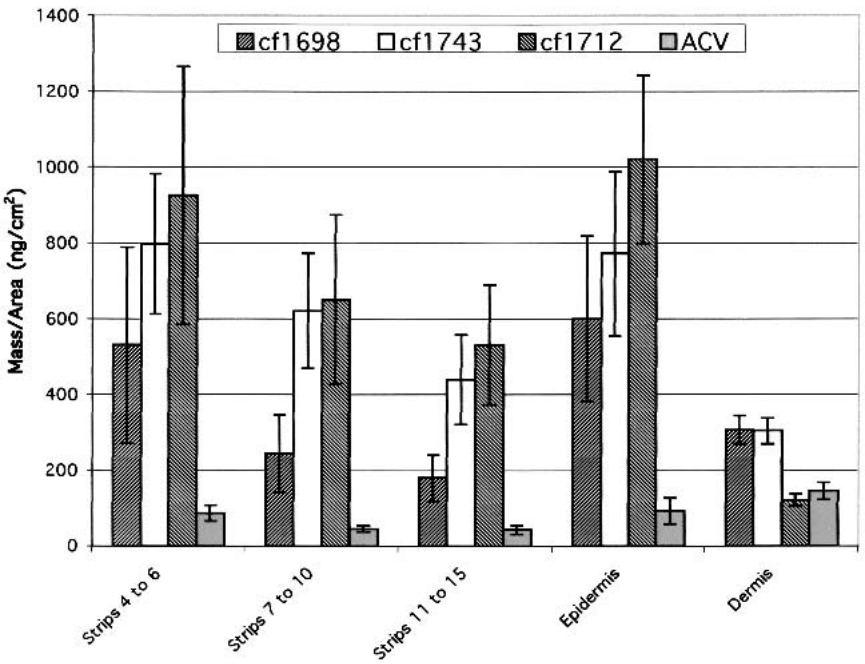

Fig. 4. Average tape stripping and extraction results after 48 hrs, focusing on stratum corneum to dermis, from cream formulations (±SEM, n = 12).

the distribution in the stratum corneum and epidermis was similar for all three Cf compounds, in the hydrophilic dermis, Cf1712 (with the longest chain) is present in the lowest concentration, showing a significantly lower partiality for the more hydrophilic environment than Cf1698 and Cf1743 exhibit. The aqueous receptor phase showed a difference again, this time with the least lipophilic Cfl698 being the only one of the three Cf compounds to leave the membrane and enter the aqueous environment. In contrast with these three compounds is the hydrophilic aciclovir, which was present at much lower concentrations in the lipophilic stratum corneum and epidermis than any of the other compounds, as might be expected. Despite its lower uptake into these lipophilic regions, it nevertheless permeated to a much greater degree than the other compounds. It seems that the aqueous receptor phase acted as an effective sink for the compound, allowing the majority of the aciclovir that reached the dermis to then dissolve in the receptor phase, removing it from the membrane. Aciclovir was not found in the dermis to a higher degree than any of the other compounds, as might have been predicted from its hydrophilicity, but once again the affinity of the compound for the water in the receptor phase is a possible explanation for this, as any aciclovir entering the dermis might quickly partition into the receptor phase. All four compounds were applied as relatively dilute creams (0.3%), and so a higher concentration of aciclovir in the donor phase might lead to higher levels of compound in the dermis, but would also be expected to show higher levels in the receptor phase.

The very different potencies of the four compounds meant that their relative concentrations in the epidermis and dermis samples do not reflect directly on how potent an antiviral effect they might elicit. Therefore the concentrations were estimated for each compound, and compared to their in vitro EC_{50} (antiviral potency) and CC_{50} (toxicity) values (Fig. 5). A log scale was used on account of the extremely low EC_{50} and high CC_{50} values determined for the test compounds (11). For these estimates, the mass of each compound per area was converted to the molar concentration per volume, with rough assumptions being made of an epidermal depth of 100 μm and a dermal depth of 1 mm (21). While the differences between most of the estimated concentrations are not particularly significant, there are significant differences between the effects they would have because of the large differences in potencies.

Cf1743 has such a low EC_{50} value that the difference between concentration required and concentration achieved at the target site is remarkable: in the epidermis the concentration achieved is around 2,000 times higher than the EC_{50}, and in the dermis it is still in the region of 45 times higher. As the target is the basal epidermis, the interface between epidermis and dermis, these findings imply that the levels reaching the target site are far higher than the EC_{50}, while remaining far lower than the minimum estimate for CC_{50}. In direct comparison with aciclovir, one of the best anti-VZV drugs currently available, in the same formulation, the molar concentrations delivered are similar, despite significant differences in absolute mass delivered. This is due to the lower molecular mass of aciclovir (see Figure 1), which means that significantly more molecules were present in the 0.3% (w/w) formulation. However, because of the enormous difference in potencies of the compounds, aciclovir is detected in the epidermis in levels that are slightly below its EC_{50}, implying that a far inferior effect might be seen. The observed delivery of Cf1743 in this formulation would be expected to translate into a safe and highly potent anti-VZV therapy, which could be several logs more efficacious than aciclovir. All of these data were seen with 0.3% creams, and it is possible that even higher concentrations could be achieved with more concentrated formulations.

The apparent retention of Cf1743 and Cf1712 in the skin is advantageous, as the purpose of these formulations was to target the active compounds to the skin, rather than the systemic circulation. As nucleoside analogues, the compounds are effective only when the virus is replicating, which occurs in the sensory ganglia at the start of reactivation, and then again when the virus reaches the skin, where it freely replicates (5). As these lipophilic compounds did not significantly

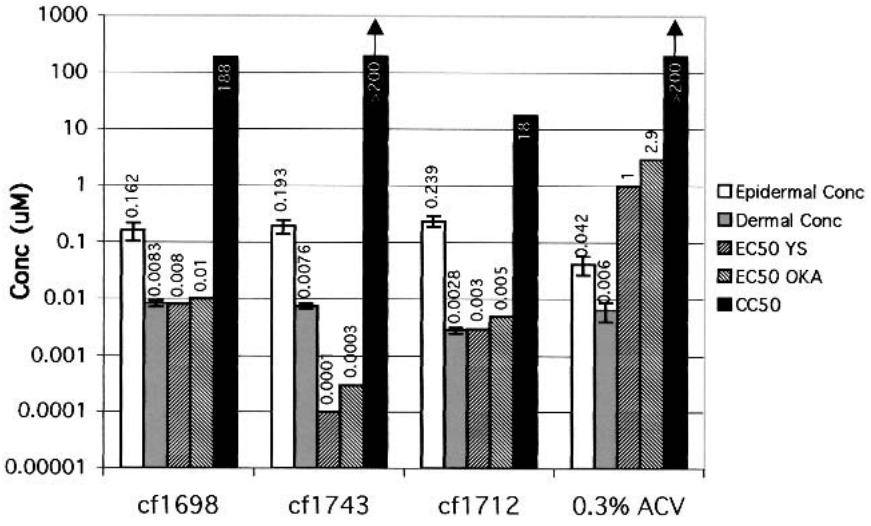

Fig. 5. Average measured epidermal and dermal concentrations (±SEM, n = 12) compared to EC_{50} values for two VZV strains (OKA and YS) and CC_{50} values, after delivery from cream formulations.

enter the receptor phase, possibly as the dermis was acting as a barrier to permeation, it implies that they would not be extensively absorbed into the bloodstream. Consequently, a greater reservoir of active compound to form in and around the target area of the skin would be expected. This would potentially provide a further advantage over hydrophilic aciclovir, which would be expected to undergo more substantial dermal clearance.

In order to reduce the incidence and extent of PHN that is suffered by shingles patients, it has been proposed that the rapid employment of effective antiviral treatment would be highly beneficial (10). The family of anti-VZV compounds discovered by McGuigan et al. (11) includes a compound that is an ideal candidate for this first-line treatment. The extremely high potency and selectivity of Cf1743 predicts that it will be a very effective drug for combating VZV, and it has been shown in this study that high concentrations can be delivered to the epidermis and dermis, the junction between the two being the region of the skin in which the virus replicates (7,22,23).

It is feasible that the application of a formulation such as the 0.3% Cf1743 cream, immediately after shingles reactivation, could rapidly and profoundly reduce the viral load and the associated inflammatory response in the infected dermatomes, thereby limiting the damage caused to the epidermal sensory nerves. By avoiding this additional and extensive nerve damage, many months or even years of pain and discomfort might be avoided, with additional savings being made by reducing the number of analgesics and non-conventional treatments that are routinely prescribed in attempts to curb the effects of PHN.

CONCLUSIONS

The data presented herein suggest that each of these novel nucleoside analogues, applied topically, could be highly efficacious in reducing the viral load of VZV in the skin and thus substantially limiting the occurrence of PHN. In particular, Cf1743 is delivered in ideal quantities to the epidermis, the area of the skin in which the virus replicates. In addition, each compound appeared to be far superior to aciclovir, the current agent of choice for VZV, in terms of the dermal and epidermal concentrations versus their in vitro activity.

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