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Formulation of cidofovir improves the anti-papillomaviral activity of topical treatments in the CRPV/rabbit model

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ABSTRACT

Current topical treatments for papillomas use ablative, cytotoxic and immunomodulating strategies and reagents. However, the effectiveness of topical treatments using different formulations has not been examined in preclinical models or clinical trials. The purpose of this study was to determine whether formulation of the small molecule acyclic nucleoside, cidofovir (CDV), could lead to improved therapeutic endpoints following topical treatment of papillomas using the cottontail rabbit papillomavirus (CRPV)/rabbit model. Different formulations with a set dose of 1% cidofovir were tested to establish comparative data.

The results demonstrated that anti-papilloma treatments with topical CDV were greatly enhanced when formulated versus unformulated. Best results were obtained with CDV formulated in cremophor, then in Carbomer 940, and then in DMSO. Further studies indicated that effective formulations led to complete cures of papillomas at dilutions less than 0.3% CDV. These studies together with previous observations demonstrated that unformulated CDV under the same treatment regime required doses of 2% to achieve cures demonstrating that much less compound can be used when properly formulated.

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1. Introduction

Currently approved topical treatments for warts include a variety of strategies such as ablation, cytotoxic reagents and immunomodulators. Ablative techniques involve curettage with scalpel, lazer or freezing (Ferenczy et al., 1995; Wollina et al., 2001; Oni and Mahaffey, 2011; Khandelwal et al., 2013); topical cytotoxic treatments include salicylic acid, trichloroacetic acid, acyclic nucleosides, podophyllotoxin, and photodynamic treatments (Snoeck, 2006); immunomodulators include interferon A, contact sensitizers such as dichlorobenzene and innate immune activators such as imiquimod (Schofer et al., 2006; Gallagher and Derkay, 2009). In general, the treatments show modest levels of efficacy (clinical outcomes summarized recently in (Kwok et al., 2012)), and include several side effects as well as recurrences (Gye et al., 2013). Improved outcomes are noted in combination treatment approaches (Kaspari et al., 2002; Lu et al., 2012; Xu et al., 2013). Thus, preclinical models to assess new and improved therapies for the treatment of HPV-associated diseases are needed, despite the existence of effective prophylactic vaccines (Kwok et al., 2012; Coremans and Snoeck, 2009).

Preclinical model systems to compare various antivirals and improved formulations are lacking, with the exception of the cottontail rabbit papillomavirus (CRPV) cutaneous wart model (Ostrow et al., 1992; Bodily et al., 1999;Christensen, 2005), the canine oral papillomavirus model (Chambers and Evans, 1959;Nicholls and Stanley, 1999) and the multi-mammate rat model (Amtmann et al., 1984; Nafz et al., 2008). We and others have used the cutaneous CRPV rabbit model extensively to examine antiviral activities (Duan et al., 2000; Kreider et al., 1992;Christensen, 2005), prophylactic and therapeutic vaccines (Breitburd et al., 1995; Jensen et al., 1997; Leachman et al., 2002) and virological studies (Hu et al., 2007). In general, the observations obtained in the rabbit model show general correlations with clinical studies (Christensen, 2005), including the phenomenon of post-treatment recurrences (Christensen et al., 2001).

Clinical trials with cidofovir (CDV) have demonstrated effectiveness against vaginal warts, skin warts and laryngeal papillomas (Van et al., 1995;De, 1996; Safrin et al., 1997; Davis et al., 2000; Snoeck et al., 2001a,b; Stragier et al., 2002;DeRossi and Laudenbach, 2004;Silverman and Pitman, 2004). The delivery strategies included topical applications in saline or gel, as well as intralesional injections. The observation of clinical recurrences

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after treatments and additional local and systemic side-effects has limited the use of this compound as a general anti-papillomavirus clinical strategy. Some of these treatment failures however may be attributable to inadequate delivery of cidofovir and the potentially short "window" of the treatments when unformulated (Snoeck et al., 2001a). Preclinical models provide opportunities to directly compare various treatment strategies that could improve clinical outcomes. Despite the existence of an effective prophylactic vaccine against several HPV types, this vaccine does not induce a post-infection therapeutic response (Munoz et al., 2009). There continues to be an unmet need for effective anti-papillomavirus treatments for existing infections and for those patients that do not receive the prophylactic vaccine. In addition, a combination of antiviral and therapeutic T-cell based vaccines may ultimately be the best strategy to cure persistent papillomavirus infections and HPV-associated precancerous lesions.

2. Materials and methods

2.1. Inoculation of rabbits with CRPV viral DNA

The studies were reviewed and approved by the Penn State University (PSU), College of Medicine IACUC, and the PSU Biological Safety and Recombinant DNA Committee. Rabbits were inoculated at 4 back sites with CRPV viral DNA using our recent delayed-scarification protocol that greatly improves the efficiency of infection with both viral DNA and infectious virions (Cladel et al., 2008). Our standard antiviral testing protocol (Christensen, 2005) is to establish two sites on the right (R) and left (L) side of rabbits with wild-type CRPV DNA (wtCRPV) and two sites on the right and left side of the rabbits with E8 mutant CRPV DNA (mE8-CRPV) (Hu et al., 2002). The latter viral genome develops papillomas that are substantially attenuated such that the papillomas are small, slow-growing and better mimic the clinical tumor mass and size of human warts. In contrast, the wtCRPV-induced sites grow rapidly and reach a diameter of 15–20 mm in 8–10 weeks (Hu et al., 2002), such that these lesions represent a significant challenge to antiviral treatments.

2.2. Treatments

The topical treatments were conducted daily for 5 days per week by applying compound in 100 μl doses onto the left-side only papillomas. Treatments began at either week two, three or four depending upon the study, and the duration of the treatments ranged from two to five weeks. The right-side papillomas were untreated and represent internal controls for the treated papillomas for each rabbit, and were compared to the placebo-treated papillomas in the control group to determine whether topically applied compounds have systemic effects. The compounds were delivered via 1 ml syringes without needles, and if formulated into a gel were gently spread over the surface of the infected area (prior to papilloma appearance) or over the surface of the papilloma (if apparent). Skin tattoo spots next to the sites of infection were used as guides to locate the original site of the viral infection for early treatments and to positionally identify sites of cures.

2.3. Formulations

Compounds were formulated as described below. A 2% stock of cidofovir in saline was prepared from which the final formulations were developed. The 1% cidofovir in saline formulation was prepared by diluting the 2% stock 1:2 with saline to make a final 1% solution. The 1% cidofovir in 10% DMSO formulation was prepared by adding the appropriate amount of DMSO and saline to achieve a final concentration of 1% cidofovir in 10% DMSO. A 2% stock of Carbomer 940 was prepared using saline, and diluted with 2% cidofovir in saline to achieve a final gel containing 1% cidofovir in 1% Carbomer 940. The final formulation was a 50:50 emulsion containing 2% cidofovir in saline and cremophor. The emulsion was prepared by mixing the solutions together using two glass syringes and a luer-lock device in a procedure often used to develop emulsions for antigen–adjuvants that use oil-based formulations. All formulations were taken up in 1 ml syringes and stored at 4 °C prior to use. Each syringe was used once for each daily group treatment.

2.4. Statistical analyses

Students *t*-test and Mann–Whitney Rank Sum test were conducted on mean papilloma sizes for treated versus untreated sites for wtCRPV and mE8-CRPV at weekly time points to determine whether significant ($p < 0.05$) differences in papilloma size were observed. The Mann–Whitney Rank Sum test was used when the data sets failed the Normality Test (Shapiro–Wilk) for the *t*-test. Graphics and statistics were conducted using SigmaPlot 11.0 software (Systat Software Inc., San Jose, CA).

3. Results

A series of experiments were conducted to test various formulations of cidofovir in the CRPV rabbit model. Cidofovir was chosen as the candidate compound as we and others have shown efficacy in the rabbit model even in the absence of any formulation (Duan et al., 2000; Christensen et al., 2000). We have observed significant activity of cidofovir in saline when daily treatments of 2% cidofovir were used topically (Christensen et al., 2000). We have also obtained therapeutic clearance of CRPV-induced papillomas by intralesional delivery of cidofovir in saline (Christensen et al., 2001). However, we noted that recurrences were common (Christensen et al., 2001), as also found in clinical treatments of both genital

Table 1
Outcome of treatments for individual sites in the formulation experiment.

Group and treatments	Papillomas induced with 5 μg wtCRPV			Papillomas induced with 5 μg mE8-CRPV		
	Growth inhibition[a]	Cures[b]	Recurrences[c]	Growth inhibition[a]	Cures[b]	Recurrences[c]
Group A (1% cidofovir in saline)	1/4	0/4	0/0	1/4	0/4	0/0
Group B (1% cidofovir in 10% DMSO)	2/4	1/4	0/1	3/4	1/4	0/1
Group C (1% cidofovir in Carbomer 940)	0/4	2/4	1/2	0/4	4/4	1/4
Group D (1% cidofovir in cremophor)	0/4	4/4	0/4	0/4	4/4	0/4
Group E (saline)	0/2	0/2	0/0	0/2	0/2	0/0
Group F (10% DMSO)	0/2	0/2	0/0	0/2	0/2	0/0
Group G (Carbomer 940)	0/2	0/2	0/0	0/2	0/2	0/0
Group H (cremophor)	0/2	0/2	0/0	0/2	0/2	0/0

[a] Papillomas per infected sites.
[b] Cured papillomas per infected sites.
[c] Recurrences per cured papillomas.

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