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The antimicrobial agent C31G is effective for therapy for HSV-1 ocular keratitis in the rabbit eye model

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ABSTRACT

The amphoteric C31G solution contains equimolar alkyl dimethlyglycine and alkyl dimethyl amine oxide buffered with citric acid. C31G acts as a broad spectrum antiviral and an antibacterial. No previous in vivo studies have been done to test C31G in an animal model of HSV-1 ocular keratitis. We assessed the anti-herpetic activity of C31G in the rabbit eye model using three treatment groups: (1) 1% trifluorothymidine (TFT); (2) 0.25% C31G plus 0.5% hydroxypropyl methylcellulose (HPMC); and (3) vehicle, 0.5% HPMC. Scarified rabbit corneas were inoculated with the HSV-1 strain McKrae. On post inoculation (PI) day 3, rabbits were placed in three balanced groups based on slit-lamp examination (SLE) scores. Treatment began on PI day 3, five times a day for five consecutive days. In addition to the daily, masked SLE scoring, the eyes were assessed daily for stromal opacity, scleral inflammation, neovascularization, eyelid inflammation, inflammatory discharge, and epiphora. C31G and TFT were very effective in reducing the lesions and pathogenesis associated with HSV-1 ocular keratitis. The vehicle control scores were significantly higher and did not effectively treat HSV-1 keratitis. C31G has the potential to be used to treat herpetic keratitis as well as other herpetic topical lesions in humans.

1. Introduction

C31G is a broad spectrum, antimicrobial and antiviral agent (Calis et al., 1992; Corner et al., 1988; Thompson et al., 1996) composed of a mixture of an alkyl dimethylglycine (alkyl betaine) and an alkyl dimethyl amino oxide, buffered with citric acid to pH 5.5–6. This unique agent is potent *in vitro* against gram negative and gram positive bacteria, enveloped viruses, and numerous fungi and yeast (including *Candida*). Since both components in C31G are amphoteric, surface active chemicals, we suggest the mechanism of action involves the binding of the polar head of the group of the amino oxide betaine mixture to the pathogen surface. This subsequently results in a disruption of the membrane by the alkyl portion of the molecules thus killing the infectious agent.

C31G, in addition to antimicrobial activity, was demonstrated in human studies to be an effective, acceptable, and safe spermicidal agent (Burke et al., 2010). Thus, C31G has dual activity, reducing

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infectivity of enveloped virus and certain other sexually transmitted disease organisms and application as a safe and well-tolerated spermicidal agent.

The present study assessed the safety and tolerance of the buffered formulation of C31G in the rabbit eye model and the eyes of squirrel monkeys. C31G has been shown to be safe and well-tolerated as a vaginal gel in humans (Burke et al., 2010) and in the pigtailed macaques (Patton et al., 2006). Patton et al. (2006) showed that 1.0% C31G gel applied to the vagina or rectum had no toxicity. However no safety and tolerance studies have been done in any eye model including rabbit or primate eyes. In addition to assessing the safety and tolerance in the rabbit and squirrel monkey eye models, the efficacy of C31G as an anti-herpetic was determined in comparison to commercial trifluorothymidine (TFT). TFT is considered by many to be the drug of choice to treat herpes keratitis in humans (Wellings et al., 1972; Coen and Richman, 2007; Hill et al., 2009). Many other anti-herpetics have been tested in the rabbit eye model (Coen and Richman, 2007; Toma et al., 2008; Webre et al., 2012). A 1% gel of C31G was tested in pigtailed macaques and found to be safe and well tolerated (Patton et al., 2006).

2. Materials and methods

2.1. Drugs, compounds, and solutions

The antiviral drug trifluorothymidine (TFT) is a 1% ophthalmic solution provided by Falcon Pharmaceutical, Fort Worth, TX. The pH was approximately 6.0 and the osmolarity is approximately 283 oMsm. The C31G was used at 0.25% in 0.5% HPMC at pH 6 provided by BioSyn Inc., Philadelphia, PA. The 0.5% HPMC at pH 6 had no C31G and was provided by Biosyn Inc., Philadelphia. Sterile balanced salt solution (BSS) at pH 6 was a laboratory preparation.

2.2. Approvals

Our studies in rabbits and primates adhered to the policies of the Association for Research and Provision in Ophthalmology (ARVO). In addition to the ARVO policies, we obtained approval for these studies from the LSUHSC Institutional Biosafety Committee (IBC) #7024 and the LSUHSC Institutional Animal Care and Use Committee (IACUC) #2850.

2.3. Animal models

2.3.1. Safety and tolerance in rabbit eyes

In vivo safety and tolerance of C31G was carried out on naïve, uninfected rabbit eyes. A series of studies was performed. Each test group had five rabbits. First, we assessed the safety and tolerance of three types of treatments in rabbit corneas with no injury for five consecutive days, five times per day. These three treatments were: (1) 0.25% C31G; (2) 0.5% HPMC at pH 6; and (3) sterile balanced salt solution (BSS) with no HPMC and no C31G. Each rabbit eye received 50 μl topically.

For the next experiment, rabbit corneas were scarified by mild crosshatch on the corneal surface. This experimental design assessed whether C31G would alter the wound healing and reepithelialization in a small crosshatch area.

A third safety and tolerance test involved removing the entire corneal epithelium in a 5 mm circle marked with a 5 mm trephine with a depth of ~0.2 mm. The three solutions noted above were used to treat the scarified rabbit eyes five times per day for five consecutive days (see Table 1).

2.3.2. Safety and tolerance in nonhuman primate eyes

We tested the three solutions in eight adult (1.2–2 kg) squirrel monkeys (*Saimiri sciureus*). These monkeys had no corneal defects. To determine any adverse effects in these primates, the right eyes were treated with 0.25% C31G in 0.5% HPMC and the left eyes were treated with BSS. This was done five times per day for five consecutive days. Slit-lamp examinations were performed each day before treatment (see Tables 1 and 2).

2.4. Virus

HSV-1 strain McKrae was used in all experiments. New Zealand white rabbit corneas were scarified in a mild crosshatch fashion. The rabbits were under general anesthesia and the corneas received topical anesthesia. Both corneas were inoculated with 50 μl of HSV-1 strain McKrae at 3 × 10^5 PFU.

2.5. HSV-1 keratitis model/chemotherapy

Three days after viral inoculation, all 48 rabbit corneas (in 24 rabbits) were scored using ocular parameters and by slit-lamp examinations with fluorescein staining (see Table 2), and then were placed into three balanced groups of eight rabbits per group. The rabbit treatment groups were (1) 0.25% C31G in 0.5% HPMC at pH 6, (2) the vehicle at 0.5% HPMC at pH 6, and (3) 1% TFT (commercially available). Topical drops (50 μl) were applied to all eyes 1.5 h apart, five times per day for five consecutive days. Administration of drugs was masked (treatment groups unknown by those giving treatments).

The slit-lamp examination and scoring of ocular parameters listed in Table 2 were marked and performed independently by two experienced corneal specialists. We have previously given details of slit-lamp procedures (Kwon et al., 1979; Majumdar et al., 2005; Hill et al., 1998; Toma et al., 2008; Webre et al., 2012).

Table 1
Outline of animal studies.

Experiment	Rabbits			Nonhuman primates	
	Group 1	Group 2	Group 3	Group 1 (Right Eyes)	Group 2 (Left Eyes)
Safety and Tolerance Normal, Naïve Corneas (No Deficit)	C31G (0.25%) + HPMC (0.5%)	HPMC (0.5%)	BSS	C31G (0.25%) + HPMC (0.5%)	BSS
Safety and Tolerance with Crosshatch of Corneal Surface	C31G (0.25%) + HPMC (0.5%)	HPMC (0.5%)	BSS	N/A	N/A
Safety and Tolerance with Corneal Debridement (5 mm circle)	C31G (0.25%) + HPMC (0.5%)	HPMC (0.5%)	BSS	N/A	N/A
Inoculation of HSV-1*	C31G (0.25%) + HPMC (0.5%)	HPMC (0.5%)	TFT (1%)	N/A	N/A

All treatments were masked. All solutions had a pH of 6.
Treatments were given at 50 μl, 5 times per day at 90 min intervals.
* Rabbits were placed into three balanced groups based on SLE scores.

Table 2
Criteria for scoring of ocular parameters.

Ocular parameters	Clinical scores			
	0 (symptomatic of naïve uninfected with no manifestations)	+1 (slight manifestations)	+2 (moderate manifestations)	+3 (severe manifestations)
Stromal opacity	No coloration, clear and transparent	Slight thickening of stromal area, translucent	Whitish-yellow coloration, thickening and scarred	Yellowish coloration of >90% of stromal area, thickening and open sores with blood patches
Scleral injection (hyperemia)	No redness, no prominent blood vessels and no chemosis	Reddish tinge, blood vessels more visible and localized chemosis	Red, prominent blood vessel network and localized chemosis	Hyperemia with individual blood vessels indistinguishable and chemosis
Ocular neovascularization	No visible blood vessels	Developing blood vessel network with <25% of the surface affected	Blood vessel network with 26–75% of the surface affected	Prominent blood vessel network with >75% of the surface vascularized
Eyelid inflammation	Eyelid margins thin with no visible redness	Eyelid margins showing localized swelling and redness	Eyelid margins showing localized swelling and visible blood vessel network	Eyelid margins swollen, red and engorged outer parts of eyelids
Inflammatory discharge	No mucous discharge	Mucous discharge collecting at the caruncle and lacrimal apparatus	Mucous discharge collecting at the caruncle, lacrimal apparatus and eyelid margin	Mucous discharge collecting at the adnexa and causing peri-ocular fur to be matted down (pseudo-membranes)
Epiphora (tears/moisture)	Moist corneal surface	Watery fluid collection mainly at the lower eyelid margin	Watery fluid collection mainly at the lower eyelid margin and wetness of the entire outer part of eyelids	Watery fluid collection mainly at the lower eyelid margin, wetness of the entire outer part of eyelids and pseudo-membranes
Slit-lamp examination (SLE) [*]	No lesions	~15–20% of corneal surface involvement, primarily dendrites	At least one geographic lesion and numerous dendrites	Mostly geographic lesions covering between 50–80% of the corneal surface

[*] SLE score of +1.5 means that at least 25% up to 40% of the cornea shows dendrites and punctate lesions but no geographic lesion.
[·] SLE score of +4.0 means that at least 90% of the corneal surface is covered with a very large geographic lesion.

Table 2 is a description of the 7 ocular clinical parameters. In addition to the 0 to +4 analyses of the corneal lesions by slit-lamp examination, the severity of ocular disease was assessed by evaluating six other clinical parameters: stromal opacity, scleral injection, eyelid inflammation, ocular neovascularization, inflammatory discharge, and epiphora (excessive tearing). Means and standard error of the means of the clinical scores were calculated daily for post inoculation (PI) days 3–7, 7.5, and 10. On PI day 7, scoring was done once before the first treatment and once after the last treatment. All the clinical parameters were masked and assessed. Ocular parameter scores of the three treatment groups are provided for comparison (See Fig. 1 and Table 2).

2.6. Statistics

Our statistical analysis was by a full non-parametric repeated mean as described by Dmitrienko et al. (2007) and Brunner et al. (2001).

3. Results

Safety and tolerance tests found that C31G was safe and well-tolerated in both rabbit and squirrel monkey eyes. Based on these experiments, we proceeded with a similar C31G dose and frequency of administration in rabbits displaying HSV-1 keratitis. Table 1 outlines these animal studies.

In the first phase of the safety and tolerance in naïve rabbit eyes, the three drugs noted in Table 1 were given to rabbits five times per day for five consecutive days and rabbits were scored. Ocular parameters and scoring criteria are given in Table 2. Safety and tolerance studies showed no adverse effects in any clinical parameter scored in any eye in any rabbit.

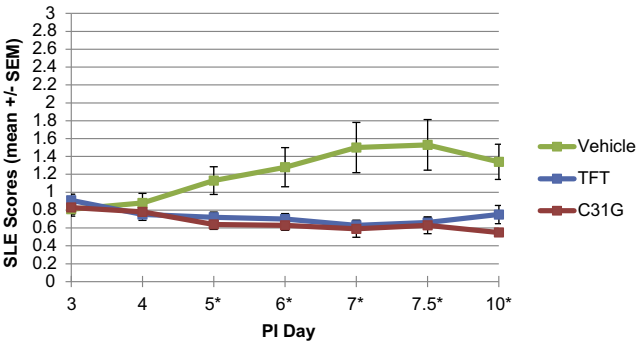
The second phase of safety and tolerance was a mild crosshatch of the corneal surface that mimics the viral inoculation procedure. These were masked analyses and all 5 rabbits (10 eyes) in each group healed completely within 72 h. We continued treatment until PI day 7 and there were no observable adverse effects in any of the six parameters listed in Table 2.

The most important test of safety and tolerance involved the debridement of the corneal epithelium to evaluate both epithelialization and migration. This mimics an infectious geographic lesion. Three groups noted in Table 1 were employed. Again, the reepithelialization was almost complete (~85%) at 72 h and was fully completed by day 4 (94 h) in all eyes in all groups. The experiment was continued for a fifth day and no adverse effects were observed.

We then conducted safety and tolerance testing without injury on normal naïve squirrel monkey eyes. This was the same dosing frequency as in rabbits, but without any injury to the corneal epithelium. Over the five days, the monkeys were examined by fluorescein staining and SLE, and there were no differences in safety and tolerance between the C31G in HPMC and the BSS. This demonstrated that C31G in HPMC is safe and well-tolerated in the non-human primate eye.

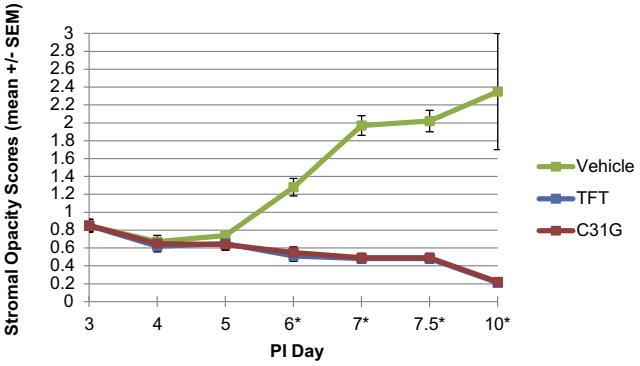
Fig. 1A shows the average corneal slit-lamp examination (SLE) scores of the three treatment groups, beginning on post-inoculation (PI) day 3. This scoring continued through PI day 10. During the first phase of treatment, there were no statistical differences (PI day 4). In the second phase of treatment, Fig. 1A shows C31G and TFT scores significantly ($p < 0.005$) lower than those from the 0.5% HPMC group. This statistical difference continued from PI day 5 through PI day 10.

Another characteristic noted in the SLE scoring is that eyes treated with C31G had geographic lesions (+2 or greater) in two of 112 observations, while eyes treated with the TFT had 3 geographic lesions out of 112 observations, and eyes treated with vehicle had



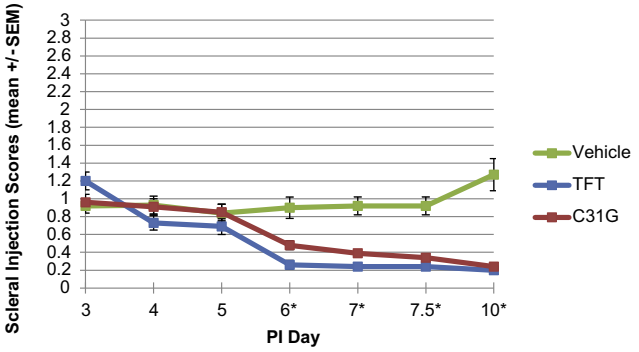
(A) SLE Scores in HSV-1 Epithelial Keratitis in NZW Rabbits

*TFT and C31G significantly different from Vehicle



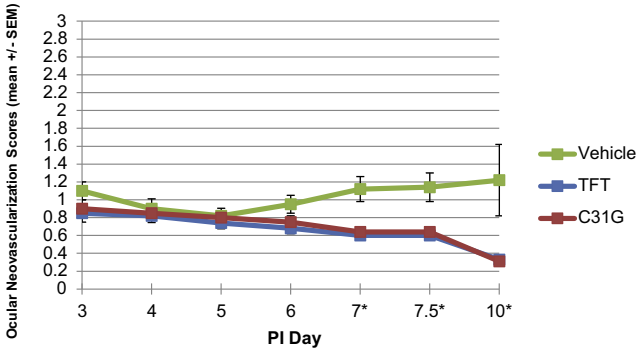
(B) Assessment of Stromal Opacity

*TFT and C31G significantly different from Vehicle



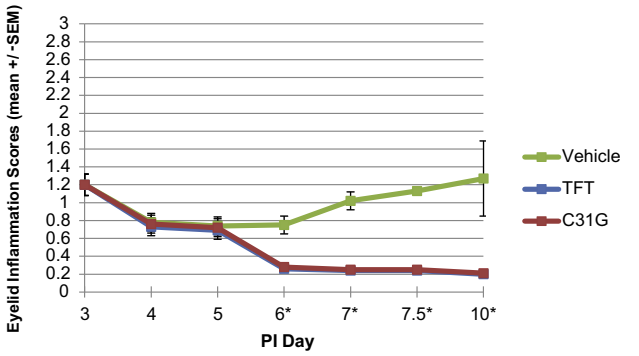
(C) Assessment of Scleral Injection

*TFT and C31G significantly different from Vehicle



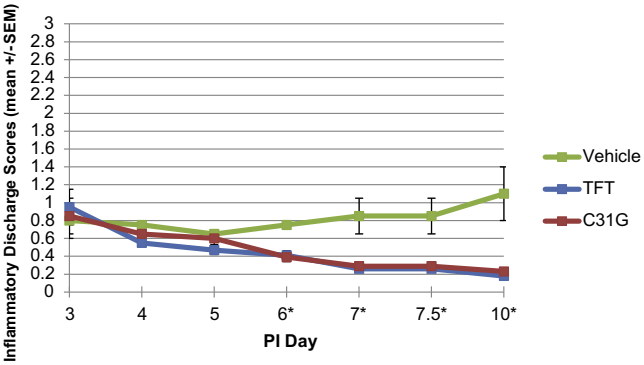
(D) Assessment of ocular neovascularization

*TFT and C31G significantly different from Vehicle



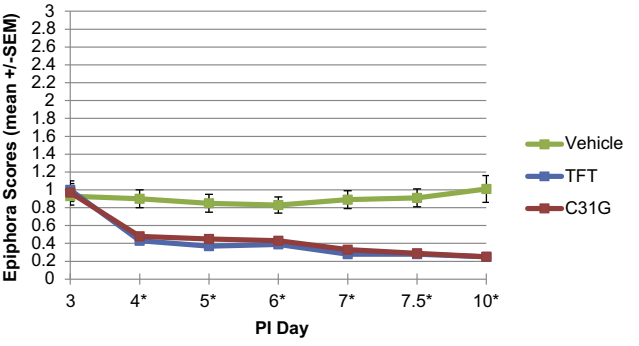
(E) Assessment of eyelid inflammation

*TFT and C31G significantly different from Vehicle



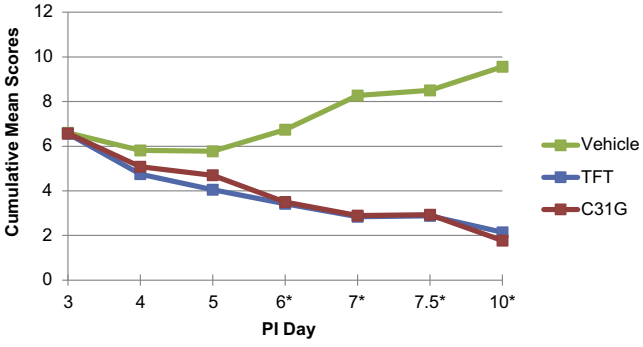
(F) Assessment of inflammation discharge (mucous)

*TFT and C31G significantly different from Vehicle



(G) Assessment of epiphora

*TFT and C31G significantly different from Vehicle



(H) Cumulative Scores of 6 Ocular Parameters

*TFT and C31G significantly different from Vehicle

Fig. 1. Assessments of 7 ocular parameters.

geographic lesions in 14 of 112 observations. This is another indicator of the efficacy of the two drugs compared to the vehicle. Most studies done on HSV-1 keratitis in animal models and patients emphasize masked slit-lamp examination scores as the most relevant. However, because this study was employing a new drug, we thoroughly assessed other ocular parameters for both safety and tolerance and efficacy in treatment.

Fig. 1B–G are of the individual ocular parameters graphed like those in Fig. 1A. Fig. 1B is the assessment of the stromal opacity. The vehicle treated eyes had significantly ($p < 0.005$) increased stromal opacity scores beginning at PI day 6 after initiation of treatments. The stromal opacity remained constant for the duration of treatments with C31G and TFT. Stromal opacity continues to increase in the 0.5% HPMC group up to PI day 10. Stromal opacity, especially when scores are 1 or greater, is irreversible and is a significant contributor to decreased vision.

Fig. 1C is the assessment of scleral injection (bloodshot eyes). The treatments with C31G and TFT were both significantly ($p < 0.005$) decreased as compared to the control, and the decline in score began on PI day 6 and continued until PI day 10. All treatments were stopped at PI day 7.

Corneal neovascularization is a major problem in acute and recurrent ocular herpes. In animal models, this condition often occurs late relative to the initial appearance of corneal lesions. Fig. 1D provides scores for corneal neovascularization in the 3 treatment groups. This parameter was significantly ($p < 0.005$) lower on PI day 7 for both the C31G and TFT groups as compared to the vehicle group. Corneal neovascularization, similar to an increase in stromal opacity, is often irreversible if not aggressively treated in patients and is a significant factor leading to blindness.

Fig. 1E is the assessment of eyelid inflammation. A significant ($p < 0.005$) reduction in eyelid inflammation began for the C31G and TFT treatment groups on PI day 6. This parameter remained constant through PI day 10, even though treatment stopped at the end of PI day 7. Fig. 1E shows that scores remained high for the vehicle group.

Fig. 1F is the assessment of inflammatory discharge (primarily mucous). C31G and TFT were effective beginning on PI day 4, 24 h after initiating treatment. The scores for C31G and TFT were significantly ($p < 0.005$) lower than the vehicle.

Fig. 1G is the scores of assessment of epiphora (tears/fluid). This was the most rapid response of the six (6) ocular parameters assessed. This response appeared 24 h after initiation of treatment and continued until PI day 10. At PI days 4–10, C31G and TFT showed significantly less ($p < 0.005$) epiphora than the vehicle group.

Fig. 1H is a cumulative representation of six ocular parameters indicated in Table 2, excluding the corneal epithelium scores (Fig. 1A). The data in Fig. 1H show that 48 h after treatment initiation (PI day 5), a statistical difference ($p < 0.005$) was observed between the vehicle group compared to the TFT and C31G groups, and this difference continued to PI day 10.

4. Discussion

A major health problem in industrialized countries, especially the USA, is the increased incidence of acyclovir-resistant and multi drug-resistant strains of HSV-1 in patients with ocular herpes infections (Webre et al., 2012). Most of the drug resistance is related to the herpes thymidine kinase. We know of no resistance to C31G in any infectious organism. Although the exact mechanism of action is not known, it may to involve pore formation disrupting the viral envelope.

Many antivirals are nucleoside analogs (Coen and Richman, 2007). A few potent antivirals are non-nucleoside analogs (Coen and Richman, 2007; Himaki et al., 2012). Compounds such as plant extracts have been reported to exhibit anti-herpetic activity *in vitro* and some *in vivo* (Nakama et al., 2012). This is due, in part, to the disruption of the lipid envelope of herpesviruses. Also, numerous proteins (Brandt et al., 2007), peptides (Bultmann and Brandt, 2002; Jose et al., 2013) such as the apolipoprotein E mimetic peptide (Bhattacharjee et al., 2008, 2009) and amphoteric compounds (Bultmann and Brandt, 2002) have been reported to exhibit anti-herpetic activity in animal models. Other novel inhibitors have been suggested to block herpes acute infections (Mulik et al., 2012) and viral reactivation epigenetically (Knipe et al., 2013; Kristie, 2012; Liang et al., 2013a,b).

C31G has been reported to have antibacterial, antifungal, and antiviral activity *in vitro* (Wyrick et al., 1997; Corner et al., 1988; Howett et al., 1999; Malamud et al., 1998; Thompson et al., 1996). There are no reported antiherpetic studies of C31G *in vivo*. C31G exhibits potent spermicidal activity and is known to penetrate cervical mucosa (Thompson et al., 1996). C31G was found safe and well-tolerated in the rabbit eye model, and allowed assessment of chemotherapeutic efficacy for HSV-1 keratitis. We evaluated C31G compared to TFT (a potent anti-herpetic agent) and to a vehicle with the same ionic strength and pH as C31G. We found C31G and TFT to have comparable activity. However, C31G is more economical and may be able to inactivate most enveloped viruses, including HIV (Krebs et al., 1999). Furthermore, C31G's mechanism of action is likely to be active in the treatment of HSV-2.

We assessed the corneal epithelium by slit-lamp examination (SLE) and six (6) other ocular parameters known to be associated with HSV-1 lesions and pathogenesis. C31G and TFT both increased healing and decreased the ocular parameters directly associated with herpes keratitis. C31G was proven safe, tolerable, and efficacious at treating herpes keratitis. It is more economical than comparable treatments (TFT) and has no known resistant organisms. Thus, we propose that C31G has promising potential for treatment of ocular herpes keratitis.

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