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INVITED REVIEW FOR *JOPT'S* 30th ANNIVERSARY YEAR

Peptide Therapeutics for Treating Ocular Surface Infections

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Abstract

Microbial pathogens—bacteria, viruses, fungi, and parasites—are significant causes of blindness, particularly in developing countries. For bacterial and some viral infections a number of antimicrobial drugs are available for therapy but there are fewer available for use in treating fungal and parasitic keratitis. There are also problems with current antimicrobials, such as limited efficacy and the presence of drug-resistant microbes. Thus, there is a need to develop additional drugs. Nature has given us an example of 1 potential source of new antimicrobials: antimicrobial peptides and proteins that are either present in bodily fluids and tissues constitutively or are induced upon infection. Given the nature of peptides, topical applications are the most likely use to be successful and this is ideal for treating keratitis. Such peptides would also be active against drug-resistant pathogens and might act synergistically if used in combination therapy. Hundreds of peptides with antimicrobial properties have been isolated or synthesized but only a handful have been tested against ocular pathogens and even fewer have been tested in animal models. This review summarizes the currently available information on the use of peptides to treat keratitis, outlines some of the problems that have been identified, and discusses future studies that will be needed. Most of the peptides that have been tested have shown activity at concentrations that do not warrant further development, but 1 or 2 have promising activity raising the possibility that peptides can be developed to treat keratitis.

Introduction

Ocular surface infections are a significant cause of blindness and can be caused by bacteria, such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Streptococcus pneumoniae*[1]; viruses, such as herpes simplex, adenovirus, and vaccinia virus[2–4]; fungal species, such as *Candida*, *Fusarium*, *Aspergillis*, and *Curvularia*; as well as parasites, such as *Acanthamoeba*.[5] Antimicrobial agents are approved for use in treating ocular infections with these pathogens, with the exception of adenovirus, but there are a number of issues with their use, including drug resistance, the need for long-term treatment, lack of efficacy, and toxicity. Because of these issues, there is a need for improved therapeutics. Antimicrobial peptides (AMPs) have significant potential for use as antimicrobial agents for ocular or other infections.[6,7] For some agents, such as *P. aeruginosa* and herpes simplex virus (HSV), a considerable amount of work has been done examining the potential therapeutic use of peptides and proteins, but for other pathogens much less has been done.

Nature Provides Examples

Nature provides us with numerous examples of the use of peptides and proteins with antimicrobial properties either present in tears or synthesized by conjunctival and corneal cells. These include the small peptides α- and β defensins and LL-37, a 37-amino-acid peptide derived from the protein hCAP18, and proteins, such as Lysozyme, Lactoferrin, Lactoferricin B, and Mucins. Several recent reviews of the presence and potential role of these peptides and proteins in ocular infections have been published so only a summary will be provided.[7–9] Briefly, α-defensins are synthesized by neutrophils infiltrating ocular tissues, while the β-defensins and hCAP18 (LL-37) are synthesized by corneal and conjunctival epithelial cells and cells in the lacrimal system. For the β-defensins some are constitutively made, while others are inducible in the context of an infection. Mucins on the corneal surface also have antimicrobial activity and appear to provide a significant barrier to infection, as evidenced by the need to scarify the cornea to achieve reproducible infections in animal models. Several proteins present in tears

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have antimicrobial activity, including Lysozyme, Lactoferrin, Lipocalin, and Secretory IgA. Proteins, such as secretory phospholipase A_2, secretory leukocyte protease inhibitor, and surfactant protein D, are also synthesized in corneal and conjunctival epithelium and play important roles in defense against infection. In addition to these secreted molecules, corneal and conjunctival cells also express pathogen-associated molecular pattern (PAMP) receptors, such as Toll-Like Receptors. Inflammasome components are important in upregulating expression of defensive molecules, such as Interferon, and several chemokines and cytokines that in turn either have direct inhibitory activity or enhance the immune response.[10] PAMPs and inflammasomes have been shown to help protect against ocular infection.[7–9]

Several different mechanisms of action are used by these natural antimicrobial agents.[7–9] The defensins and LL-37, which are cationic, have the potential to form pores in bacterial membranes resulting in cell death. The antifungal activity of defensins and LL-37 may also be related to pore formation. The defensins and LL-37 can bind to viruses and either prevent attachment, block entry, or both. This activity includes nonenveloped viruses where it appears that the peptides inhibit attachment and/or entry or can disrupt capsids. One caveat is that the activity of some defensins is reduced at salt concentrations present in tears.[11] In addition, the constitutively synthesized defensins are present at concentrations below those necessary to kill bacteria, but may achieve effective concentrations following induction during an infection. Lysozyme digests bacterial and possibly fungal cell walls. The chelation of iron is responsible for some of the antibacterial and antifungal activities of Lactoferrin but it also inhibits viral infections by interfering with attachment, entry, or both. The antiviral activity is most likely due to a highly basic sequence at the N-terminus (lactoferricin). Lipocalin interferes with microbial siderophores responsible for transport of iron into microbes and deprivation of iron inhibits growth. Secretory phospholipase A_2 cleaves lipid molecules in bacterial membranes, thereby disrupting the cells, and secretory leukocyte protease inhibitor may prevent the activity of damaging microbial proteases. Secretory IgA binds to pathogens and prevents attachment, neutralizes infectivity, can bind to lectin-like molecules on pathogens facilitating removal, and has chemotactic activity for neutrophils.

Studies on the role of these peptides and proteins in protecting ocular surfaces have had mixed results.[8] *In vitro* studies have shown that these natural AMPs and proteins have broad-spectrum inhibitory activity and they are produced constitutively or are induced upon infection.[12–14] Studies that used knockout or naturally mutant mice, antibody depletion, or si/shRNA-knockdown approaches have shown that some of the AMPs appear to be protective.[15–19] Due to redundancy, single-knockout studies, animal models may not be an accurate indicator that a particular peptide or protein is not important. Multiple AMPs and enzymes are present in tears and on the corneal surface and the activities of these could mask the loss of a single component. In addition, there are synergistic interactions between components that could affect the interpretation of single-knockout studies. As pointed out by Kolar and McDermott[8] and Silva et al.,[7] the full potential of naturally occurring AMPs and proteins has not been adequately explored.

Several approaches have been used in developing and testing peptides for potential efficacy in treating microbial keratitis. The more traditional approach involves identifying peptides (ie, AMPs) with direct antimicrobial activity. Such studies involve *in vitro* testing of the test articles for either inhibition of growth [eg, minimum inhibitory concentration (MIC) values] or microbicidal activity that directly kills the pathogen, followed, in some cases, by testing potential efficacy in an appropriate animal model. *In silico* methods to design optimal peptides have also been used, as well as phage display strategies. Animal models exist for the most common bacterial pathogens—fungi, *Acanthamoeba*—and for HSV-1 and vaccinia infections. Rabbits and mice are the most frequently used animals and infections either involve corneal scarification followed by topical application of the microbe, stromal injection of the pathogen, or corneal abrasion. The most common treatment modality is topical application of the test article in an aqueous solution, such as phosphate-buffered saline (PBS) or artificial tears; in a gel formulation involving a thickening excipient; or in a cream. More hydrophobic test articles are usually given in cream formulations.

In this review I focus primarily on peptides that have been tested *in vivo* for efficacy in treating ocular pathogens, but will mention some more recent peptides that have only been tested *in vitro*. Several peptides have been tested *in vitro* against ocular isolates (reviewed in Mannis[20]). Some of these represent naturally occurring molecules but several are synthetic peptides. In general, the quality and completeness of the studies vary widely and more rigor is needed in studies testing AMPs. Many of the peptides and proteins that have been studied have moderate efficacy but a few of them appear to be as effective as currently available drugs and have significant promise for clinical use. Table 1 summarizes the peptides that have been tested only *in vitro* and Table 2 lists those peptides that have been tested in animal models.

Five general approaches have been used for peptides that have direct antimicrobial effects. These include AMPs themselves without modification, AMPs with specific modifications designed to enhance the activity, synthetic peptides, peptides derived from functional domains of proteins, and peptide-drug conjugates. Of the hundreds of AMPs that have been identified,[21] only a handful have been tested in cell culture for potential utility in treating ocular infections and even fewer have been adequately tested in animal models.

Antimicrobial Peptides

Unmodified AMPs

An AMP from the King Cobra, OH-CATH30, was shown to inhibit 10 clinical isolates of *P. aeruginosa* with MIC values for most strains at a concentration of $\sim 6\,\mu g/mL$ and had synergistic activity with antibiotics that inhibit topoisomerase.[22] Studies in a rabbit model showed that OH-CATH30 alone was very effective in preventing corneal clouding and reduced the bacterial burden by ~ 100-fold. In addition, inflammatory infiltrates in the cornea were reduced as were the amounts of proinflammatory cytokines. *In vivo* toxicity studies were not included but this study is very encouraging and may lead to the clinical use of this AMP.

TABLE 1. PEPTIDES TESTED *IN VITRO* FOR EFFICACY AGAINST OCULAR PATHOGENS

Test article	*Chemical nature*	*Tested in*	*Source*	*Target microbe*	*Concentration tested*	*Effect*	*References*
Gomesin	Peptide	Cell culture	*Acanthoscurria gomesiana*/synthetic	*Acanthamoeba castellanii*	0–64 μM	Reduced growth	60
Tryptophan-tagged AMPs	Peptide	Contact lenses	Synthetic	*Pseudomonas aeruginosa*	2.5–320 μM	90% reduction in bacterial survival	29
Pc-C and Pc-E	Peptides	Corneal epithelial cells	Phage display synthetic	*Aspergillus fumigatis*	0–100 μM	Reduced binding to cells	40
LL-37	Cathelicidin	Cell culture	Human/synthetic	Adenovirus, HSV-1	EC_{50} 118–270 μM[a]	Reduced viral replication, blocks attachment	48,49
P5 and P6	Peptides	Cell culture	Trialisyn/synthetic	*A. castellanii*	0–100 μM	P5 inhibited growth but SI was 0.6	60
$CAP37_{20–44}$	Peptide	Cell culture	Synthetic	Bacteria, *Candida*; adenovirus, HSV-1	50–750 μg/mL	Inhibits infection, kills pathogen	56–58
GRDSP	Peptide	Cell culture	Integrin binding/synthetic	adenovirus	0–1 mg/mL	Reduced replication	48

[a]Dependent on adenovirus type.
AMP, antimicrobial peptide; HSV, herpes simplex virus; SI, specificity index.

AMPs have also been tested for viral infections. The circular θ defensins are not synthesized by humans but are present in Old World Monkeys, gibbons, and orangutans and have been shown to have broad-spectrum antiviral activity, including HSV.[23–26] We tested the potential efficacy of a θ defensin (RC-2) in a mouse model of HSV-1 keratitis.[27] In yield reduction assays with HSV-1 KOS, the EC_{50} value was ~10 μM (20 μg/mL). For the mouse study, we used PBS with or without 2% methylcellulose (gel) at a concentration of 0.1%. RC-2 significantly reduced keratitis scores when used prophylactically (preincubation of virus with RC-2 in PBS or topical application in the gel) but was not effective when treatment was initiated between 3 and 4 h postinfection. With postinfection treatment viral shedding from the cornea was reduced by ½ log unit, which was significant, but not as effective as Viroptic[28]; thus, RC-2 may not be worth further study for HSV keratitis.

Modified AMPs

Over many years numerous modifications have been made in AMPs for structure–function analysis and to potentially improve the activity, reduce toxicity, or improve pharmacokinetic parameters. Unfortunately only a few of these have been tested for efficacy in keratitis models. Using the rationale that AMPs target bacterial membranes, Pasupuleti et al.[29] tested the effect of adding tryptophan tags to peptides from kininogen to improve the membrane interaction. They found that potency *in vitro* increased as the length of the tag increased with MIC values of 2.5 μM against *P. aeruginosa* with a 5-residue tag. They then incubated the peptide with contact lenses that were seeded with bacteria and showed that bacterial counts were reduced by ~90%. Thus this peptide may be useful in lens-cleaning solutions. To my knowledge these peptides have not been tested in actual corneal infection models.

Nos-Barbera et al.[30] synthesized a hybrid peptide containing amino acids from cecropin A and melittin and tested their activity against *P. aeruginosa*. The MIC values ranged from 1 to 4 μg/mL. They then applied peptide topically (0.1%) in a rabbit model of keratitis using 2 treatment regimens—6 instillations every 2 h and 12 installations applied hourly—and scored clinical disease. They found that with either regimen there was a ~30% to 40% reduction in the severity of keratitis, which was comparable to the effect of Gentamicin. This work does not appear to have been followed up with further development perhaps because efficacy did not appear to be significantly better than the antibiotic and the peptide would be more costly.

Synthetic peptides

Mannis[20] used a computational drug design strategy to identify potential AMPs. These peptides—designated CCIA, B, and C and Col-1—were tested *in vitro* against ocular isolates of *P. aeruginosa*, *S. aureus*, and *Staphylococcus epidermidis* and found that they reduced colony counts by 2 to 3 logs depending on the bacterium and the peptide. They then tested the Col-1 peptide in a rabbit model of *P. aeruginosa* keratitis. The peptides were delivered in 0.5 methylcellulose/0.05% EDTA at concentrations of 10 or 50 μg/mL. The treatment regimen was 1 drop every 15 min for the first hour followed by 9 additional drops at hourly intervals

Table 2. Peptides Tested *In Vivo* for Efficacy Against Ocular Pathogens

Test article	*Chemical nature*	*Tested in*	*Source*	*Target microbe*	*Concentration tested*	*Vehicle*	*Model used*	*Effect*	*References*
OH-CATH30	Peptide	Rabbits	King Cobra	*P. aeruginosa*	1 mg/mL	PBS	Abrasion	Reduced colony counts	22
RC-2	Theta defensin	Mouse	Nonhuman primate/ synthetic	HSV-1	0.1% (wt/vol)	DMEM	Scarification	Reduced disease severity when used prophylactically	27
Cecropin/Melitin	Hybrid peptide	Rabbit	Cecropia moth/ bee	*P. aeruginosa*	0.1%	PBS	Intrastromal injection	Modest reduction in disease severity	30
Nona-D-arginine	Peptide	Mouse	Synthetic	*P. aeruginosa*	10 and 100 μM	PBS	Scarification	Reduced colony counts	38,39
CC1A, B, C, and Col-1	Peptides	Rabbit	*In silico* designed synthetics	*P. aeruginosa*	10 and 50 μg/mL	0.5 methylcellulose ± 0.05% EDTA	Abrasion	Not effective	20
G1 and G2	Peptides	Mouse	Phage display synthetics	HSV-1	0.5 mM	PBS	Scarification	Reduced viral replication and less-severe disease	32
Pc-C and Pc-E	Peptides	Mouse	Synthetic	*A. fumigatis*	1 mg/mL	PBS	Scarification	Reduced growth, less-severe disease	40
TAT-Cd^{o}	Peptide	Mouse	HIV tat protein/ synthetic	HSV-1	1%	PBS; tears naturale; 2% methylcellulose in PBS; Aquaphor based cream	Scarification	Reduced angiogenesis and stromal disease	46
apoEdp	Peptide	Mouse/rabbit	Apolipoprotein E/synthetic	HSV-1	0.5 mM	PBS	Scarification	Very effective in reducing shedding and disease	54–55
G2-ACV	Peptide-nucleoside analog conjugate	*Ex vivo* porcine cornea	Synthetic	HSV-1	10 μM	Normal saline	Scarification	Reduced viral antigen in tissue—not better than ACV alone	68

ACV, Acyclovir; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline.

for the first day. On days 2–4 one drop was given every hour for 10 h each day. Treatment was initiated 12–14 h postinfection. Unfortunately, Col-1 treatment did not result in significantly reduced keratitis scores but did reduce the bacterial load at 96 h postinfection. Although delaying the onset of treatment for several hours more closely mimics a patient setting, this might have accounted for the lack of effect *in vivo*.

Several cationic peptides have been shown to have antimicrobial activity most likely by binding to surface structures on the microbes and inhibiting their function. For example, cationic peptides prevent HSV-1 attachment and/or entry blocking the infection.[26,31,32] All d-polyarginines inhibit the protease furin that is responsible for cleavage and activation of *P. aeruginosa* exotoxin A and are a significant virulence factor.[33–37] Based on these observations Karicherla and Hobden tested the efficacy of a nona-D-arginine peptide in a mouse model of *P. aeruginosa* infection.[38,39] They found a dose-dependent significant reduction in keratitis scores in mice treated with either 10 or 100 μM peptide. Treatment with peptide and ciprofloxacin reduced keratitis scores below that of either peptide or antibiotic alone and the levels of corneal cytokines were reduced in treated eyes. Although they found significant reductions in disease, the keratitis was not completely prevented so it is not clear that this peptide would be useful clinically.

Tiwari et al.[32] screened a M13 phage peptide display library for peptides that bound to heparan sulfate (HS) and 3-OS HS. The rationale being that HSV-1 initially binds to HS on the cell surface and 3-OS HS is a coreceptor for HSV-1 glycoprotein that is required for entry into cells. They identified 2 peptides (G1 and G2) from this screen that inhibited HSV-1 entry with EC_{50} values of 20–30 μM. Both G1 and G2 inhibited several strains of virus. They then showed that, when given prophylactically (0.5 mM) on mouse corneas, infection and keratitis severity were significantly reduced. To date, G1 and G2 have not been tested in a postinfection treatment regimen. The peptides are cationic and structure–function analysis indicated that positively charged residues were important; thus, these peptides may be acting in a manner similar to the TAT-Cd^o peptide we have worked with and other cationic peptides, although the EC_{50} for TAT-Cd^o is lower.

Using a similar phage display approach, Zhao et al.[40] isolated 2 peptides, PC-C and PC-E, that inhibited *Aspergillis* adhesion to human corneal stromal cells with EC_{50} values in the range of 3–5. These peptides also inhibited adhesion to mouse corneas when added at a concentration of 100 μM one hour prior to addition of the fungus. They also tested Pc-C and Pc-E in a mouse corneal scarification model and found that colony forming units were reduced by 50% one day postinfection and significantly reduced keratitis scores on days 3 and 5 after infection.

Peptides derived from proteins

Most peptides are poorly taken up by eukaryotic cells so most development efforts of peptide antimicrobials have focused on topical uses and generally target surface structures on the pathogens. However, a number of peptides are now known that can facilitate cellular uptake of moieties coupled to them. In an effort to get an antiviral peptide into cells we decided to couple a peptide that blocked HSV ribonucleotide reductase (RR)[41,42] to these ''cell penetrating peptides'' (CPPs) and tested their activity against HSV-1. To our surprise, we found that the CPPs themselves had antiviral activity[43,44] that was sometimes better than the RR-coupled peptides. One of the peptides was a 13-residue peptide derived from the HIV-1 tat protein that was shown to inhibit HSV-1 infection of corneal epithelial cells.[45] We subsequently modified the tat peptide by adding a C-terminal cysteine, amidating the C-terminal carboxyl group, and using all d-amino acids to reduce susceptibility to proteases (TAT-Cd^o). These changes improved the EC_{50} and gave the peptide virucidal activity.[31] We then tested the efficacy in a mouse model of herpetic keratitis using 4 different vehicles: PBS, 2% methylcellulose gel, artificial tears, and a cream.[46] We found, regardless of the formulation, that TAT-Cd^o applied topically 5 times per day at a concentration of 1 mg/mL significantly reduced the severity of corneal vascularization and stromal keratitis. Treatment reduced viral shedding by 1.5 logs at 24 h postinfection. We also showed that TAT-Cd^o was not toxic to mouse corneas.[47] These results were encouraging but the peptide was not as effective as Viroptic.

Uchio et al.[48] tested hCAP18 (LL-37) against several adenovirus strains, including those involved in epidemic keratoconjunctivitis, in cell culture and found that the EC_{50} values ranged from 118 to 270 μM. Unfortunately, these EC_{50} values suggest that this peptide should not be developed further even though the CC_{50} value was $\sim$3,200 μg/mL, because concentrations this high would be expensive and would unlikely to be effective *in vivo*. In a novel approach, Lee et al.[49] engineered corneal epithelial cells to express LL-37. They found that the secreted LL-37 inhibited binding of HSV-1 to cells but this was not sufficient to completely protect the cells. Lee et al.[49] also constructed composite nanoparticle-hydrogel corneal implants containing LL-37, and showed that these could inhibit HSV-1 attachment to cells in culture. These gels have not been tested in animal models, however.

Human apolipoprotein E allele ϵ4 has been identified as a risk factor in HSV infections and carriers of the allele have an increased risk of cold sores and genital herpes.[50–52] Dobson et al.[53] found that an apoE dimer tandem repeat peptide (apoEdp) derived from the receptor binding region of the APOE protein inhibited HSV-1 infection of cells with an EC_{50} value of 16.6 μM, HSV-2 with an EC_{50} of $\sim$40 μM, and *P. aeruginosa* with an EC_{50} value of 2.5 μM. The antiviral activity is directed toward very early events and given that the receptor binding region of ApoE is known to bind HS, the mechanism of inhibition for HSV-1 and HSV-2 is probably related to this activity. Battacharjee et al.[54] tested the efficacy of the apoEdp (1% in PBS) peptide using a mouse model of HSV-1 keratitis. The apoEdp was found to be as effective as 1% Viroptic in reducing the severity of keratitis and enhanced clearance of the virus. The expression of several cytokines associated with corneal inflammation was also reduced. This group subsequently tested the effect of apoEdp in a rabbit herpes keratitis model with results similar to those in the mouse model.[55] In the rabbit study, a TK-negative strain (resistant to nucleoside analog antivirals) was used, suggesting that peptides would be active against resistant strains. In neither of these articles was corneal toxicity evaluated but, because apoEdp was as effective as the currently approved ocular antiviral (Viroptic),

this peptide is probably worth further development. In addition, because apoEdp also inhibits *P. aeruginosa*[53] it should be tested for efficacy in bacterial keratitis models.

CAP37 is a neutrophil-derived protein that contains a 25-amino-acid sequence (residues 20–44) with antimicrobial activity. Peptides derived from CAP37 have been shown to have broad-spectrum activity against several ocular pathogens, including *P. aeruginosa* and *S. aureus*.[56] Gordon et al.[57] found that CAP37 peptides inhibited HSV-1 and some types of adenoviruses in cell culture; however, ocular types, such as Ad8 and Ad19, were not inhibited. Thus, CAP37 peptides may be useful for treating herpetic keratitis. CAP37 peptides also have inhibitory activity against several species of *Candida* so they may also be useful for treating fungal keratitis.[58] To date, there are no reports testing CAP37 peptides in animal models of ocular infection.

Adenoviruses use $\alpha5\beta1$ integrin for entry into cells via an RDG motif[59]; thus, RDG-containing peptides might interfere with this interaction. Based on this rationale, Uchio et al.[48] tested the effect of a peptide containing this motif (GRGDSP) against several strains of adenoviruses, including those that cause epidemic keratoconjunctivitis, and found that they reduced replication. However, the EC_{50} values were in the low-mM range (0.14–1.1), which is not sufficiently low to warrant further development. The peptide is very short so the reduced activity may have been due to the failure of the peptide to adopt a specific conformation needed for efficient interaction with the target, so it is possible that the activity could be improved with further work.

Sacramento et al.[60] synthesized peptides corresponding to the amino terminus of a protein called trialysin and tested them for antiparasitic activity against *Acanthamoeba castellanii*. They also tested gomesin, an AMP from spiders. The trialysin-derived peptides, denoted P5 and P6, fold into amphipathic helices and had been shown to induce bacterial and protozoan lysis.[61] Gomesin is a β-hairpin peptide and had been shown to have antibacterial and antifungal activities.[62,63] They found that the P5 peptide completely inhibited growth at a concentration of 7 μM, while P6 had little, if any, effect and gomesin required a concentration of 64 μM to inhibit growth to the same extent as P5. In parasite permeabilization assays, the 50% concentrations were 34.5, 59, and 23 μM, respectively, for P5, P6, and gomesin. However, P5 had a 50% permeabilization concentration of 22 μM yielding a therapeutic index of 0.6. This is not surprising because amphipathic peptides can be toxic to eukaryotic cells. In addition, P5 was sensitive to protease digestion that would affect the half-life in clinical use. The observation that growth inhibition by P5 occurred at a lower concentration than permeabilization suggests that additional antiparasitic mechanisms are involved. Because of the poor therapeutic index, these peptides are unlikely to be efficacious and further work is not warranted.

Peptide-drug conjugates

Eukaryotic cells express dipeptide transport proteins raising the possibility that conjugation of an antimicrobial to a dipeptide might increase cellular uptake.[64] In addition, the addition of a valine residue to acyclovir or ganciclovir to create prodrugs increases oral bioavailability.[65–67] Using this rationale, Park et al.[68] coupled acyclovir (ACV) to the 3-OS heparin sulfate binding G2 peptide mentioned previously[32] and tested it for efficacy. In a plaque reduction assay, they found that the G2-ACV was active, indicating that conjugation did not reduce the activity of ACV. Similar results were seen when *ex-vivo*-infected porcine corneas were stained for HSV antigen that was used as a surrogate for viral replication. It is not clear why they did not test for titer reductions in the *ex vivo* model or why their mouse model was not used and they did not test their hypothesis that conjugation to G2 would improve corneal uptake.

Concluding Remarks

Hundreds of AMPs have been described in the literature (reviewed in Refs.[21,69]) and numerous synthetic peptides have been described, yet only a few have been tested for their ability to inhibit common ocular pathogens and even fewer have been evaluated in animal models for efficacy. Comparing the available data, it presents a mixed picture of the clinical potential of peptide therapeutics for ocular infection. Some of the peptides tested show little or no activity, others have modest efficacy, and 1 or 2 have activity comparable to currently available small molecules. What is clear is that more peptides need to be tested in rigorous studies. In addition, there needs to be a systematic analysis of structure activity relationships with these peptides to improve their activity in the eye, identify the mechanisms whereby they inhibit specific pathogens, how structural features affect delivery to the ocular surface, and determine pharmacokinetics and optimal formulations. Although it is known that many AMPs form pores in bacterial membranes, there may be other mechanisms of inhibition that are involved. The mechanisms for inhibiting viral pathogens are different from bacteria and there is very little information on how they inhibit fungal or parasitic pathogens. It is likely that peptides will be active against pathogens resistant to currently available antimicrobials. Additionally, combinations of peptides, or combinations of peptides and small-molecule antimicrobials might show synergism and should be tested.

One factor that may reduce activity is that short peptides may be adopting random-coil structures in solution and the optimal activity requires the formation of α-helical or β-sheet structures. This issue needs systematic study in the context of the ocular surface and has been addressed for other applications through the introduction of specific amino acid sequences, circularizing peptides, or using stapled peptide approaches.[70–72]

Another critical area that has been poorly addressed is the pharmacokinetics of potentially therapeutic peptides on the ocular surface. We know very little about the residence time of peptides on the cornea and in the tear film in relation to the kinetics of washout. We know little if anything about their ability to penetrate into the cornea or whether they reach the interior of the eye. We also know very little about stability of the peptides. Cationic peptides in particular may be susceptible to proteolytic digestion. Some of the peptides that have been tested have been synthesized from all d-amino acids and are therefore resistant to digestion but it is not known for many of the peptides whether all d-versions would retain the desired activity.

A systematic study of peptide formulation for ocular delivery needs to be undertaken. Some rudimentary studies

of the activity of a peptide in different formulations have been done but most studies have simply suspended the peptide in PBS for topical delivery. Formulation with specific excipients could improve residence time on the cornea or enhance penetration that could improve the efficacy.

There is wide variation in the quality of peptide studies. Studies should include determination of EC_{50} values reported as micromolar concentrations in order for comparisons to be made with small molecules and other peptides. Every study needs to have a CC_{50} determination and a specificity index (SI or therapeutic index) needs to be calculated. Studies to determine the mechanism of action need to be included and preliminary animal efficacy studies are needed for peptides with EC_{50} values at nanomolar or low micromolar concentrations. Control groups with peptide only in uninfected eyes should be included in every animal study to provide a preliminary test of toxicity unless a more complete *in vivo* toxicity analysis is presented as a separate study. One also needs to be cognizant of the fact that unless a peptide is active in culture at nanomolar or low micromolar concentrations and has an SI value > 10 it is highly unlikely to be a viable candidate for further development. For example, the DP178 peptide (T20; Fuzeon), which is FDA approved for use in treating HIV infections, inhibits infection of T-cells with an EC_{50} of 0.001 μg/mL.[73]

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Author Disclosure Statement

Dr. Brandt has been awarded patents for peptide antivirals and they have been assigned to the Wisconsin Alumni Research Foundation.

References

1. Wilhelmus, K.R. Review of clinical experience with microbial keratitis associated with contact lenses. *CLAO J.* 13:211–214, 1987.
2. Liesegang, T.J. Epidemiology of ocular herpes simplex: natural history in Rochester, Minn. 1950 through 1982. *Arch. Ophthalmol.* 107:1160–1165, 1989.
3. Gonzalez-Lopez, J.J, Morcillo-Laiz, R., and Munoz-Negrete, F.J. Adenoviral keratoconjunctivitis: an update. *Arch. Soc. Esp. Oftalmol.* 88:108–115, 2013.
4. Pepose, J.S., Margolis, T.P., LaRussa, P., and Pavan-Langston, D. Ocular complications of smallpox vaccination. *Am. J. Ophthalmol.* 136:343–352, 2003.
5. McCulley, J.P, Alizadeh, H., and Niederkorn, J.Y. *Acanthamoeba* keratitis. *CLAO J.* 21:73–76, 1995.
6. Pereira, H.A. Novel therapies based on cationic antimicrobial peptides. *Curr. Pharm. Biotechnol.* 7:229–234, 2006.
7. Silva, N.C., Sarmento, B., and Pintado, M. The importance of antimicrobial peptides and their potential for therapeutic use in ophthalmology. *Int. J. Antmicrob. Agents* 41:5–10, 2013.
8. Kolar, S.S., and McDermott, A.M. Role of host-defence peptides in eye diseases. *Cell Mol. Life Sci.* 68:2201–2213, 2011.
9. McDermott, A.M. Antimicrobial compounds in tears. *Exp. Eye Res.* 117:53–61, 2013.
10. Pearlman, E., Sun, Y., Roy, S., et al. Host defense at the ocular surface. *Int. Rev. Immunol.* 32:4–18, 2013.
11. McDermott, A.M., Rich, D., Cullor, J., et al. The *in vitro* activity of selected defensins against an isolate of *Pseudomonas* in the presence of human tears. *Br. J. Ophthalmol.* 90:609–611, 2006.
12. Otri, M., Mohammed, I., Abedin, A., et al. Antimicrobial peptides expression by ocular surface cells in response to *Acanthamoeba castellanii*: an *in vitro* study. *Br. J. Ophthalmol.* 94:1523–1527, 2010.
13. Mohammed, I., Abedin, A., Tsintzas, K., et al. Increased expression of hepcidin and toll-like receptors 8 and 10 in viral keratitis. *Cornea.* 30:899–904, 2011.
14. Gottsch, J.D., Li, Q., Ashraf, M.F., et al. Defensin gene expression in the cornea. *Curr. Eye Res.* 17:1082–1086, 1998.
15. Wu, M., McClellan, S.A., Barrett, R.P., and Hazlett, L.D. β-defensin-2 promotes resistance against infection with *P. aeruginosa*. *J. Immunol.* 182:1609–1616, 2009.
16. Augustin, D. K., Heimer, S.R., Tam, C., et al. Role of defensins in corneal epithelial barrier function against *Pseudomonas aeruginosa* traversal. *Infect. Immun.* 79:595–605, 2011.
17. Huang, L.C., Reins, R.Y., Gallo, R.L., and McDermott, A.M. Cathelicicin-deficient (*Cnlp*-/-) mice show increased susceptibility to *Pseudomonas aeruginosa* keratitis. *Invest. Ophthalmol. Vis. Sci.* 48:4498–4508, 2007.
18. Wu, M., McClellan, S.A., Barrett, R.P., Zhang, Y., and Hazlett, L.D. β-defensins 2 and 3 together promote resistance to *Pseudomonas aeruginosa* keratitis. *J. Immunol.* 183:8054–8060, 2009.
19. Kolar, S.S., Baidouri, H., Hanlon, S., and McDermott, A.M. Protective role of murine β-defensins 3 and 4 and cathelin-related antimicrobial peptide in *Fusarium solani* keratitis. *Infect. Immun.* 81:2669–2677, 2013.
20. Mannis, M.J. The use of antimicrobial peptides in ophthalmology: an experimental study in corneal preservation and the management of bacterial keratitis. *Trans. Am. Ophthalmol. Soc.* 99:243–271, 2001.
21. Bulet, P., Stocklin, R., and Menin, L. Antimicrobial peptides: from invertebrates to vertebrates. *Immunol. Rev.* 198:169–184, 2004.
22. Li, S.-A., Liu, J., Xiang, Y., et al. Therapeutic potential of the antimicrobial peptide OH-CATH30 for antibiotic-resistant *Pseudomonas aeruginosa* keratitis. *Antimicrob. Agents Chemother.* 58:3144–3150, 2014.
23. Nguyen, T.X., Cole, A.M., and Lehrer, R.I. Evolution of primate theta-defensins: a serpentine path to a sweet tooth. *Peptides.* 24:1647–1654, 2002.
24. Cole, A.M., Hong, T., Boo, L.M., et al. Retrocyclin: a primate peptide that protects cells from infection by T- and M-tropic strains of HIV-1. *Proc. Natl. Acad. Sci. U. S. A.* 99:1813–1818, 2002.
25. Leikina, E., Delanoe-Ayari, H., Melikov, K., et al. Carbohydrate-binding molecules inhibit viral fusion and entry by cross-linking membrane glycoproteins. *Nat. Immunol.* 6:995–1001, 2005.

26. Yasin, B., Wang, W., Pang, M., et al. Theta defensins protect cells from infection by herpes simplex virus by inhibiting viral adhesion and entry. *J. Virol.* 78:5147–5156, 2004.
27. Brandt, C.R., Akkarawongsa, R., Altmann, S., et al. Evaluation of a θ-defensin in a murine model of herpes simplex virus type 1 keratitis. *Invest. Ophthalmol. Vis. Sci.* 48:5118–5124, 2007.
28. Brandt, C.R., Coakley, L.M., and Grau, D.R. A murine model of herpes simplex virus-induced ocular disease for antiviral drug testing. *J. Virol. Methods.* 36:209–222, 1992.
29. Pasupuleti, M., Chalupka, A., Morgelin, M., Schmidtchen, A., and Malmsten, M. Tryptophan end-tagging of antimicrobial peptides for increased potency against *Pseudomonas aeruginosa*. *Biochim. Biophys. Acta.* 1790:800–808, 2009.
30. Nos-Barbera, S., Portoles, M., Morilla, A., et al. Effect of hybrid peptides of cecropiin A and melittin in an experimental model of bacterial keratitis. *Cornea.* 16:101–106, 1997.
31. Bultmann, H., Teuton, J., and Brandt, C.R. Addition of a C-terminal cysteine improves the anti-herpes simplex virus activity of a peptide containing the human immunodeficiency virus type 1 tat protein transduction domain. *Antimicrob. Agents Chemother.* 51:1596–1607, 2007.
32. Tiwari, V., Liu, J., Valyi-Nagy, T., and Shukla, D. Anti-heparan sulfate peptides that block herpes simplex virus infection *in vivo. J. Biol. Chem.* 286:25406–25415, 2011.
33. Sarac, M.S., Cameron, A., and Lindberg, I. The furin inhibitor hexa-D-arginine blocks the activation of *Pseudomonas aeruginosa* exotoxin A *in vivo*. *Infect. Immun.* 70:7136–7139, 2002.
34. Hazlett, L.D., Berk, R.S., and Iglewski, B. H. Microscopic characterization of ocular damage produced by *Pseudomonas aeruginosa* toxin A. *Infect. Immun.* 34:1025–1035, 1981.
35. Miyazaki, S., Matsumoto, T., Ohno, A., and Yamaguchi, K. Role of exotoxin A in inducing severe *Pseudomonas aeruginosa* infections in mice. *J. Med. Microbiol.* 43:169–175, 1995.
36. Inocencio, N.M., Moehring, J.M., and Moehring, T.J. Furin activates *Pseudomonas* exotoxin A by specific cleavage *in vivo* and *in vitro*. *J. Biol. Chem.* 269:31831–31835, 1994.
37. Pillar, C.M., and Hobden, J.A. *Pseudomonas aeruginosa* exotoxin A and keratitis in mice. *Invest. Ophthalmol. Vis. Sci.* 43:1437–1444, 2002.
38. Karicherla, P., and Hobden, J.A. Nona-D-arginine therapy for *Pseudomonas aeruginosa* keratitis. *Invest. Ophthalmol. Vis. Sci.* 50:256–262, 2009.
39. Karicherla, P., Aras, S., Aiyar, A., and Hobden, J.A. Nona-D-arginine amide (D9R) suppresses corneal cytokines in *Pseudomonas aeruginosa* keratitis. *Cornea.* 29:1308–1314, 2010.
40. Zhao, G., Li, S., Zhao, W., et al. Phage display against corneal epithelial cells produced bioactive peptides that inhibit *Aspergillus* adhesion to the corneas. *PLoS One.* 7:e33578, 2012.
41. Dutia, B., Frame, M.C., Subak-Sharpe, J.H., Clark, W.N., and Marsden, H.S. Specific inhibition of herpes virus ribonucleotide reductase by synthetic peptides. *Nature.* 321:439–441, 1986.
42. Cohen, E.A., Gandreau, P., Brazeau, P., and Langelier, Y. Specific inhibition of herpes virus ribonucleotide reductase by a nonapeptide derived from the carboxy terminus of subunit 2. *Nature.* 321:441–443, 1986.
43. Bultmann, H., Busse, J.S., and Brandt, C.R. Modified FGF4 signal peptide inhibits entry of herpes simplex virus type 1. *J. Virol.* 75:2634–2645, 2001.
44. Bultmann, H., and Brandt, C.R. Peptides containing membrane-transiting motifs inhibit viral entry. *J. Biol. Chem.* 277:36018–36023, 2002.
45. Larsen, I.V., and Brandt, C.R. A cationic tat peptide inhibits herpes simplex virus infection of human corneal epithelial cells. *J. Ocul. Pharmacol. Ther.* 26:541–547, 2010.
46. Jose, G.G., Larsen, I.V., Gauger, J., et al. A cationic peptide, TAT-Cd^{o}, inhibitis herpes simplex virus type 1 ocular infection *in vivo*. *Invest. Ophthalmol. Vis. Sci.* 54:1070–1079, 2013.
47. Akkarawongsa, R., Cullinan, A.E., Zinkel, A., Clarin, J., and Brandt, C.R. Corneal toxicity of cell-penetrating peptides that inhibit herpes simplex virus entry. *J. Ocul. Pharmacol. Ther.* 22:279–289, 2006.
48. Uchio, E., Inoue, H., and Kadonosono, K. Anti-adenoviral effects of human cationic antimicrobial protein-18/LL37, an antimicrobial peptide, by quantitative polymerase chain reaction. *Korean J. Ophthalmol.* 27:199–203, 2013.
49. Lee, C.-J., Buznyk, O., Kuffova, L., et al. Cathelicidin LL-37 and HSV-1 corneal infection: peptide versus gene therapy. *Trans. Vis. Sci. Technol.* 3:4, 2014.
50. Lin, W.R., Graham, J., MacGowan, S.M., Wilcock, G.K., and Itzhaki, R. Alzheimer's disease, herpes virus in brain, apolipoprotein E4 and herpes labialis. *Alzheimers Rep.* 1:173–178, 1998.
51. Hill, J.M., Battacharjee, P.S., and Neumann, D.M. Apolipoprotein E alleles can contribute to the pathogenesis of numerous clinical conditions including HSV-1 corneal disease. *Exp. Eye Res.* 84:801–811, 2007.
52. Bhattacharjee, P.S., Neumann, D.M., Foster, T.P., et al. Effect of human apolipoprotein E genotype on the pathogenesis of experimental ocular HSV-1. *Exp. Eye Res.* 87:122–130, 2008.
53. Dobson, C.B., Sales, S.D., Hoggard, P., Wozniak, M.A., and Crutcher, K.A. The receptor-binding region of human apolipoprotein E has direct anti-infective activity. *J. Infect. Dis.* 193:442–450, 2006.
54. Battacharjee, P.S., Neumann, D.M., Foster, T.P., et al. Effective treatment of ocular HSK with a human apolipoprotein E mimetic peptide in a mouse eye model. *Invest. Ophthalmol. Vis. Sci.* 49:4263–4268, 2008.
55. Battacharjee, P.S., Neumann, D.M., and Hill, J.M. A human apolipoprotein E mimetic peptide effectively inhibits HSV-1 TK-positive and TK-negative acute epithelial keratitis in rabbits. *Curr. Eye Res.* 34:99–102, 2009.
56. Pereira, H.A., Erdem, I., Pohl, J., and Spitznagel, J.K. Synthetic bactericidal peptide based on CAP37: a 37kDA human neutrophil granule-associated cationic antimicrobial protein chemotactic for monocytes. *Proc. Natl. Acad. Sci. U. S. A.* 65:2803–2811, 1993.
57. Gordon, Y.J., Romanowski, E.G., Shanks, R.M.Q., et al. A. CAP37-derived antimicrobial peptides have *in vitro* antiviral activity against adenovirus and herpes simplex virus type-1. *Curr. Eye Res.* 34:241–249, 2009.
58. Pereira, H.A., Tsyshevskaya-Hoover, I., Hinsley, H., et al. Candidacidal activity of synthetic peptides based on the antimicrobial domain of the neutrophil-derived protein, CAP37. *Med. Mycol.* 48:263–272, 2010.
59. Huang, S., Kamata, T., Takada, Y., Ruggeri, Z.M., and Nemerow, G.R. Adenovirus interaction with distinct integrins mediates separate events in cell entry and gene delivery to hematopoietic cells. *J. Virol.* 70:4502–4508, 1996.

60. Sacramento, R.S., Martins, R.M., Miranda, A., et al. Differential effects of α-helical and β-hairpin antimicrobial peptides against *Acanthamoeba castellanii*. *Parasitology* 136:813–821, 2009.
61. Martins, R.M., Sforca, M.L., Amino, R., et al. Lytic activity and structural differences of amphipathic peptides derived from trialysin. *Biochemistry.* 45:1765–1774, 2006.
62. Silva Jr., P.I., Daffre, S., and Bulet, P. Isolation and characterization of gomesin, an 18-residue cysteine rich defense peptide from the spider *Acanthoscurria gomesiana* hemocytes with sequence similarities to horshoe crab antimicrobial peptides of the tachyplesin family. *J. Biol. Chem.* 275:33464–33470, 2000.
63. Mandard, N., Bulet, P., Caille, A., Daffre, S., and Vovelle, F. The solution structure of gomesin, an antimicrobial cysteine-rich peptide from the spider. *Eur. J. Biochem.* 269:1190–1198, 2002.
64. Lee, V.H. Membrane transporters. *Eur. J. Pharm. Sci.* Suppl 2:S41–S50, 2000.
65. Anand, B.S., Nashed, Y., and Mitra, A.K. Novel dipeptide prodrugs of acyclovir for ocular herpes infections: bioreversion, antiviral activity, and transport across rabbit cornea. *Curr. Eye Res.* 26:151–163, 2003.
66. Majumdar, S., Nashed, Y.E., Patel, K., et al. Dipeptide monoester ganciclovir prodrugs for treating HSV-1 induced corneal epithelial and stromal keratitis: *in vitro* and *in vivo* evaluations. *J. Ocul. Pharmacol. Ther.* 21:463–474, 2005.
67. Weller, S., Blum, M.R., Doucette, M., et al. Pharmacokinetics of the acyclovir pro-drug valaciclovir after escalating single-and multiple-dose administration to normal volunteers. *Clin. Pharmacol. Ther.* 54:595–605, 1993.
68. Park, P.J., Antoine, T.E., Farooq, A.V., Valyi-Nagi, T., and Shukla, D. An investigative peptide-acyclovir combination to control herpes simplex virus type 1 ocular infection. *Invest. Ophthalmol. Vis. Sci.* 54:6373–6381, 2011.
69. Ganz, T. Defensins and other antimicrobial peptides: a historical persepective and an update. *Comb. Chem. High Throughput Screen.* 8:209–217, 2005.
70. Henchey, L.K., Jochim, A.L., and Arora, P.S. Contemporary strategies for the stabilization of peptides in the α-helical conformation. *Curr. Opin. Chem. Biol.* 12:692–697, 2008.
71. Huang, Y., Huang, J., and Chen, Y. Alpha-helical cationic antimicrobial peptides: Relationships of structure and function. *Protein Cell.* 2:143–152, 2010.
72. Huang, Y., He, L., Li, G., et al. Role of helicity of α-helical antimicrobial peptides to improve specificity. *Protein Cell.* 5:631–642, 2014.
73. Wild, C., Greenwell, T., et al. The inhibitory activity of an HIV type 1 peptide correlates with its ability to interact with a leucine zipper structure. *AIDS Res. Hum. Retroviruses.* 11:323, 1995.

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