Potential of ceragenin CSA-13 and its mixture with pluronic F-127 as treatment of topical bacterial infections

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Abstract

Aims—Ceragenin CSA-13 is a synthetic mimic of cationic antibacterial peptides, with facial amphiphilic morphology reproduced using a cholic acid scaffold. Previous data have shown that this molecule displays broad-spectrum antibacterial activity, which decreases in the presence of blood plasma. However, at higher concentrations, CSA-13 can cause lysis of erythrocytes. This study was designed to assess *in vitro* antibacterial and haemolytic activity of CSA-13 in the presence of pluronic F-127.

Methods and Results—CSA-13 bactericidal activity against clinical strains of bacteria associated with topical infections and in an experimental setting relevant to their pathophysiological environment, such as various epithelial tissue fluids and the airway sputum of patients suffering from cystic fibrosis (CF), was evaluated using minimum inhibitory and minimum bactericidal concentration (MIC /MBC) measurements and bacterial killing assays. We found that in the presence of pluronic F-127, CSA-13 antibacterial activity was only slightly decreased, but CSA-13 haemolytic activity was significantly inhibited. CSA-13 exhibits bacterial killing activity against clinical isolates of *Staphylococcus aureus*, including methicillin-resistant strains, *Pseudomonas aeruginosa* present in CF sputa, and biofilms formed by different Gram (+) and Gram (−) bacteria. CSA-13 bactericidal action is partially compromised in the presence of plasma, but is maintained in ascites, cerebrospinal fluid, saliva, and bronchoalveolar lavage fluid. The synergistic action of CSA-13, determined by the use of a standard checkerboard assay, reveals an increase in CSA-13 antibacterial activity in the presence of host defence molecules such as the cathelicidin LL-37 peptide, lysozyme, lactoferrin and secretory phospholipase A (sPLA).

Conclusion—These results suggest that CSA-13 may be useful to prevent and treat topical infection.

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Significance and Impact of the Study—Combined application of CSA-13 with pluronic F-127 may be beneficial by reducing CSA-13 toxicity.

Keywords

antibacterial agents; microbiological assay; pluronic F-127

Introduction

The ubiquity of natural cationic antibacterial peptides (CAPs) and the variation in amino acid sequences among CAPs suggest that facial amphiphilicity, rather than specific biochemical interactions with a unique bacterial target, defines the mechanisms by which they exert bactericidal effects. This concept has motivated many efforts to design and synthesize steroid conjugate mimics of CAPs that, in addition to having CAP-like biochemical properties, are characterized by lower cost of synthesis, stability under physiological conditions and resistance to protease digestion (Malabarba *et al.* 1992; Li *et al.* 1999; Randazzo *et al.* 2009). CSA-13 is a leading molecule from the ceragenin family tested to date and is relatively simple to prepare and purify (Epand *et al.* 2008; Lai *et al.* 2008). CSA-13 displays antimicrobial activity against vancomycin- resistant *Staphylococcus aureus* (VRSA) (Chin *et al.* 2007), multidrug-resistant *Pseudomonas aeruginosa* (Chin *et al.* 2008), *Helicobacter pylori* (Leszczynska *et al.* 2009) and periodontopathic bacteria such as *Streptococcus mutans* and *Porphyromonas* species (Isogai *et al.* 2009). CSA-13 is also active against vaccinia virus (VV) (Howell *et al.* 2009) and *Trypanosoma cruzi* (Lara *et al.* 2009). In animal studies, CSA-13 shows low toxicity, supporting this compound's possible application in human treatment (Saha *et al.* 2008). However, similar to CAPs (Wang *et al.* 1998; Ciornei *et al.* 2005), CSA-13 antibacterial activity was found to be compromised in the presence of human blood plasma (Bucki *et al.* 2007b). This finding might partially explain why CSA-13 was not effective when applied as a conjugate in polyurethane foam pads to prevent a peri-implant tissue infection that mimics contaminated osseointegrated implants in a sheep model (Perry *et al.* 2009).

Apart from being antimicrobial, CSA-13 binds and neutralizes bacterial lipopolysaccharide (Bucki *et al.* 2007b) and in combination with dioleoylphosphatidylethanolamine (DOPE), facilitates the uptake of a reporter plasmid into various cell lines (Kichler *et al.* 2005). CSA-13 might therefore have beneficial effects in the treatment of sepsis and has potential for application in gene therapy. The mechanism of CSA-13 action is not fully understood, but it involves direct interaction with negatively charged bacterial membrane molecules such as phosphatidylglycerol, which results in membrane permeabilization and depolarization (Lai *et al.* 2008; Van Bambeke *et al.* 2008).

Pluronic F-127 (poloxamer 407) is a nonionic surfactant composed of polyoxyethylene–polyoxypropylene copolymers characterized by low toxicity and good solubilizing capacity (Escobar-Chavez *et al.* 2006). At a concentration up to 10^{-4}%, pluronic F-127 forms mono-molecular micelles and at a concentration above, multimolecular aggregates consisting of a hydrophobic central core with hydrophilic polyoxyethylene chains facing the external medium (Escobar-Chavez *et al.* 2006). The unique thermoreversible and drug release characteristics of F-127 show great promise for its application as a pharmaceutical vehicle for drug delivery. Accordingly, effective treatment of chronic otitis caused by methicillin resistant *Staph. aureus* (MRSA) was achieved using a temperature responsive pluronic F-127 / vancomycin matrix system (Lee *et al.* 2004). Additionally, in the previous study, pluronic F-127 was shown to increase the incorporation of acetoxymethyl ester dye into cells (Maruyama *et al.* 1989), the parenteral delivery of peptides and proteins from its co-formulated nanoparticles (Barichello *et al.* 1999), and was identified as an important

component of a bioengineered skin graft which holds great promise for improving the healing of skin defects (Cai *et al.* 2005).

In this study, we report that CSA-13 is highly active against clinical isolates of bacteria causing different topical infections and bacterial biofilms. CSA-13 haemolytic activity decreases in the presence of pluronic F-127, and its antibacterial activity is potentiated in the presence of host defence antibacterial molecules. The results suggest that cholic acid-based mimics of CAPs, such as CSA-13, have potential for broad-spectrum treatment of bacteria associated with topical infection.

Methods

Materials

Brain heart infusion agar (BHI) was from Emapol (Gda sk, Poland). Pseudomonas isolation agar, tryptic soy broth (TSB), Brucella broth (BB) and Mueller Hinton Agar (MHA) were purchased from DIFCO (Sparks, MD, USA). An ID 32 STAPH kit to identify staphylococcal isolates and Mannitol salt agar (MSA) were from bioMérieux, (La Balme Les Grottes, France), E-tests to determine susceptibility to methicillin were obtained from AB Biodisk (Solna, Sweden). Beta-lactamase (cefinase) test was from Becton Dickinson (San Jose, CA, USA). *Pseudomonas aeruginosa* Xen 5 strain engineered through conjugation and transposition of a plasmid carrying the transposon Tn*5 luxCDABE* was purchased from Caliper Life Science Inc. (Mountain View, CA, USA). LL-37 peptide was purchased from Peptide 2.0 Inc. (Chantilly, VA, USA). Pluronic F-127, lactoferrin, lysozyme and secretory phospholipase A (sPLA) were from Sigma (St Louis, MO, USA). CSA-13 was synthesized as described previously (Ding *et al.* 2002). Cystic fibrosis (CF) sputum samples were collected from patients attending the University of Pennsylvania Health System Adult Cystic Fibrosis Center at Presbyterian Hospital (PA, USA; IRB-803255).

Haemolytic and bactericidal activities of CSA-13 in the presence of pluronic F-127

CSA-13 haemolytic activity was tested using red blood cells (RBCs) suspended in phosphate-buffered saline (PBS) (haematocrit *c.* 5%) with and without the addition of pluronic F-127 (1 or 5%). RBCs were incubated for 1 h at 37°C after CSA-13 addition (0– 500 μmol l^{-1}). Relative haemoglobin concentration in supernatants after centrifugation at 2000 g was monitored by measuring optical absorbance at 540 nm. Then, 100% haemolysis was taken from samples in which 2% Triton X-100 was added. CSA-13 bactericidal activity against *Ps. aeruginosa* Xen 5 and clinical isolate *Staph. aureus* A1 was determined as described previously (Bucki *et al.* 2004; Bucki and Janmey 2006). *Pseudomonas aeruginosa* Xen 5 and *Staph. aureus* A1 were grown to mid-log phase at 37°C (controlled by the evaluation of optical density at 600 nm) and resuspended in PBS. Two different experiments were performed to assess CSA-13 antibacterial activity. In the first, CSA-13 bactericidal activity was evaluated using a bacterial killing assay. When required, pluronic F-127 was added to the bacterial suspension before CSA-13 treatment, and after 1-hour incubation at 37°C bacterial suspensions were placed on ice and diluted 10-to 1000-fold. Then, 10 μl aliquots of each dilution were spotted on *Pseudomonas* isolation agar or LB agar. The number of colonies at each dilution was counted the following morning. The colony-forming units (CFU ml^{-1}) of the individual samples were determined from the dilution factor. In a second experiment, changes in *Ps. aeruginosa* Xen 5 chemiluminescence signals during a 5-min period were evaluated after CSA-13 (10 μmol l^{-1}) addition to its TSB suspension (*c.* 10^8 CFU ml^{-1}) using a FlexStation 3 platform (Molecular Devices, Sunnyvale, CA, USA) or after 50 s and 16 h using a Fuji Film LAS-300 system.

Antimicrobial activity of CSA-13 against clinical bacterial strains

Bacteria from clinical specimens were grown on MSA. *Staphylococcus aureus* identification was performed with an ID 32 STAPH kit followed by reading of results using the ATB system bioMérieux, according to the manufacturer's instructions. Susceptibility to methicillin and vancomycin and the presence of beta-lactamase were determined using E-test and cefinase respectively. *Staphylococcus aureus* susceptibility to macrolides, lincosamides and streptogramins B was evaluated using diffusion methods on MHA with bacterial inoculum density at 0·5 (McFarland scale) (Fiebelkorn *et al.* 2003; Standards CLSI 2009). CSA-13 minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) against different strains of *Staph. aureus* (8×10^5 CFU ml^{-1}) were determined using Mueller–Hinton broth and MHA respectively. The bactericidal activity of CSA-13 against *Ps. aeruginosa* in sputum samples collected from CF patients with chronic lung infection was measured as previously described (Tang *et al.* 2005; Bucki *et al.* 2007a). The CF sputa were diluted 10×, and 100 μl samples were treated with antibacterial agents (10–50 μmol l^{-1}) with and without pluronic F-127 (1 or 5%). After 1 h of incubation at 37°C, the suspensions were placed on ice and diluted 10- to 1000-fold. Then, 10-μl aliquots of each dilution were spotted on *Pseudomonas* isolation agar plates for overnight culture at 37°C. The number of colonies at each dilution was counted the following morning. The colony-forming units (CFU ml^{-1}) of the individual samples were determined from the dilution factor.

CSA-13 activity against bacterial biofilm

Surface hydrophobicity of *Ps. aeruginosa* Xen 5, *Moraxella catarrhalis*, *H. pylori*, *Staph. aureus* ATCC43300 and *Enterococcus faecalis* was estimated using the salt aggregation test (SAT) (Lindahl *et al.* 1981). Biofilm biomass of bacteria after CSA-13 treatment was evaluated by an optical density reading at 450 nm. Bacterial survival in the biofilm following exposure to the antibiotic was assessed after triphenyltetrazolium chloride (TTC) addition (viable bacteria can reduce the TTC to a red product depending on the level of dehydrogenase activity) (Bartoszewicz *et al.* 2007). For all bacterial strains (except *H. pylori*), biofilm formation was performed in aerobic conditions using polystyrene plates with bacterial inocula at *c.* 10^8 CFU ml^{-1} in TSB. *Helicobacter pylori* biofilm formation was performed in BB suspension under microaerophilic conditions maintained with the use of a Gas Pack-Campylobacter gas generation kit BR60 (Oxoid, Cambridge, UK). For each experiment, bacterial suspensions were placed in polystyrene plates, and a biofilm was allowed to form for 24–72 h. Plate adherence ability was used to differentiate between biofilm and planktonic bacteria that were washed out before adding CSA-13 to individual wells. In the case of *Ps. aeruginosa* Xen 5, in addition to OD measurement, biofilm biomass was evaluated using crystal violet (CV) staining (0·1%) (Peeters *et al.* 2008) and chemiluminescence intensity measurements (as an additional determinant of biofilm viability), which was evaluated using a Fuji Film LAS-300 system before and after CSA-13 treatment. Chemiluminescence (densitometry analysis) was performed using Image Gauge (version 4.22) software (Fuji Photo Film Co, Edison, NJ, USA).

Antibacterial activity of CSA-13 in different body fluids

Many factors present at infection sites such as negatively charged glycosaminoglycans, mucins, DNA, and F-actin can compromise host antibacterial peptide activity as well as that of their synthetic mimics (Weiner *et al.* 2003; Herasimenka *et al.* 2005). Recently, we found that CSA-13 resists inhibitory action of molecules present in CF sputum (Bucki *et al.* 2007b) and saliva (Bucki *et al.* 2008), and CSA-13 activity in other body fluid has not been studied yet. To assess CSA-13 potential to kill bacteria in different environments, we evaluated changes of *Ps. aeruginosa* Xen 5 luminescence (*c.* 10^8 CFU ml^{-1}) in PBS or PBS mixed

with 50% blood plasma, ascites, cerebrospinal fluid, saliva or BAL at different time points, up to 6 h after CSA-13 addition (10 and 30 μmol l^{-1}).

CSA-13 antibacterial activity in combination with host defence molecules

Combined antibacterial activity was determined with the use of a standard checkerboard assay (Rahbar *et al.* 2010; Norden 1982). A serial dilution of two antibacterial agents mixed together in microtiter plates, so that each row and column of the plate contained a fixed amount of one agent, and an increasing concentration of a second one was prepared before bacteria addition. The concentration ranged from approximately the MIC value to an eightfold dilution below this amount. Each plate also contained a row and column in which a serial dilution of each agent was presented alone. A calculation of fractional inhibitory concentration (FIC) index (FCI$_{index}$ = FIC$_A$ + FIC$_B$ = [A] /MIC$_A$ + [B] /MIC$_B$) was used to determine synergy (Kristiansen 1992).

Statistical analysis

Data are reported as mean ± SD from 3 to 6 experiments. Differences between means were evaluated using the unpaired Student's *t*-test, with $P < 0.05$ being taken as the level of significance.

Results

Pluronic F-127 decreases haemolytic but not antibacterial activity of CSA-13

It is generally accepted that CAPs and their mimics possess a mechanism of antibacterial action that involves nonspecific interference with plasma membrane lipid organization, which results in the loss of the membrane's ability to control homoeostasis of the intracellular environment. This mechanism assures a large spectrum of antibacterial functions mediated by these molecules, but on the other hand, can result in cytotoxicity towards host cells. Evaluation of haemoglobin release from erythrocytes (RBC) provides a simple measure of potential cytotoxicity for this class of molecules. As shown in Fig. 1, CSA-13 does not affect RBC membrane permeability at concentrations sufficient to kill different bacteria (Fig. 1b,c, Tables 1 and 2). However, at a concentration 10× higher than average MBC values, against different clinical strains of *Staph. aureus* (Table 1), we observed a concentration-dependent increase in haemoglobin release (Fig. 1a). Notably, in the presence of pluronic F-127, CSA-13 haemolytic activity was greatly reduced. More precisely, in combination with 5% pluronic F-127, a concentration of ceragenin CSA-13 of 100 μmol l^{-1}, which by itself had caused 20% of RBC lysis, becomes nonhaemolytic. As shown in Fig. 1b,c, the killing activity of CSA-13 against *Ps. aeruginosa* Xen 5 and *Staph. aureus* A1 evaluated in PBS enriched with 1 and 5% pluronic acid F-127 did not show significant differences compared to the killing activity of the control.

CSA-13 antibacterial activity against clinical isolates of *Staphylococcus aureus*

Bacteria from the *Staphylococcus* group can cause a multitude of diseases as a result of infection in various tissues. However, very often the infections caused by *Staph. aureus* take place on the surface of skin. Susceptibility testing of different clinical isolates including MRSA, MLS$_B$ and β-lactamase (+) *Staph. aureus* strains ($n = 16$) demonstrated that the MIC and MBC values were on average 1·27 ± 0·62 and 4·58 ± 1·75 μmol l^{-1} and range between 0·8–3·125 and 1·56–6·25 μmol l^{-1} respectively (Table 1). On a molar base, these values were at least two times lower compared to antibacterial activity of cathelicidin LL-37 against the same strains (data not shown).

Outgrowth of *Pseudomonas aeruginosa* bacteria from cystic fibrosis sputum samples treated with CSA-13 or its combination with pluronic F-127

Chronic airway infection is associated with abbreviated life spans of patients suffering from cystic fibrosis lung disease. A limited number of types of organisms are responsible for these infections, with *Ps. aeruginosa* and *Staph. aureus* being of primary importance (Gilligan 1991; Mainz *et al.* 2009). As shown in Table 2, the number of *Ps. aeruginosa* colonies outgrowing from CSA-13 (1–50 μmol l^{-1}) treated sputum samples was lower compared to control. Inhibition of bacterial outgrowth was also observed after CSA-13 treatment in combination with pluronic acid F-127. However, in two of six study samples (samples 1, 5; Table 2), 50 μmol l^{-1} of CSA-13 in combination with 5% pluronic F-127 had a smaller effect than that of CSA-13 by itself. This effect might indicate the ability of pluronic F-127 to promote bacterial growth of some *Ps. aeruginosa* strains.

CSA-13 activity against bacterial biofilm

Bacteria in a biofilm state represent a high morbidity risk due to antibiotic resistance. Chemiluminescent signals generated by *Ps. aeruginosa* Xen 5, CV staining and OD measurements accompanied by the evaluation of bacterial ability to reduce TTC generated data indicating strong antibacterial biofilm activity of ceragenin CSA-13 (Fig. 2). Addition of CSA-13 to a *Ps. aeruginosa* Xen 5 suspension resulted in a quick (*c.* 50 s) decrease of chemiluminescent signals, indicating that CSA-13 is able to reach and interfere with the metabolic machinery of bacterial cells on the order of seconds. On the long term, this interference resulted in bacterial death as indicated by the lack of detectable chemiluminescent signal after 16 h of incubation (see inset in panel a of Fig. 2). This effect was confirmed by a control experiment in which there was no bacterial outgrowth from the *Ps. aeruginosa* Xen 5 samples treated with CSA-13 or polymyxin B after 16 h of incubation (data not shown). As indicated by data in Fig. 2b, CSA-13 is not only active against planktonic *Ps. aeruginosa* Xen 5, but it can effectively kill bacteria embedded in biofilm. Anti-biofilm activity of CSA-13 is maintained in the presence of pluronic F-127. Additionally, as shown in Fig. 2c, CSA-13 is effective against biofilm formed by *Mor. catarrhalis*, *H. pylori*, *Staph. aureus* ATCC43300 and *Ent. faecalis.*

CSA-13 activity in various body fluids

Antibacterial activity of exogenous molecules can be modulated by different host factors present at infection sites. To assess the potential of CSA-13 to kill bacteria in various clinical settings, we evaluated the luminescence of *Ps. aeruginosa* Xen 5 bacteria in PBS suspensions containing 50% of various body fluids including plasma, ascites, cerebrospinal fluid, saliva and BAL (Fig. 3). All specimen collection was performed in accordance with a protocol approved by the Medical University of Białystok Ethics Committee for Research on Humans and Animals, and written consent was obtained from all subjects. In accordance with the data shown in Fig. 2a, decreased *Ps. aeruginosa* Xen 5 luminescence by CSA-13 is indicative of bactericidal activity. The increased bacterial luminescence that was observed 6 h after bacterial growth in the presence of different body fluids, compared to luminescence in PBS, indicates differences in bacterial growth that likely reflect the availability of nutrition and the variable concentration of endogenous host immune defence molecules. Under 10 μmol l^{-1} CSA-13 treatment, a decrease of bacterial luminescence was observed in all evaluated body fluids, but to different extents. The least amount of activity (a drop of 33% in luminescence) was observed in the presence of human plasma. With an increase in CSA-13 concentration from 10 to 30 μmol l^{-1}, luminescence decreased significantly for all body fluids. More precisely, luminescence decrease was 66, 93, 96, 98 and 99% respectively, in plasma, ascites, cerebrospinal fluid, saliva and BAL. These data suggest that the antibacterial activity of CSA-13 is compromised in blood plasma, which might limit CSA-13 use for systemic application, but remains high in other biofluids.

CSA-13 activity in the presence of LL-37 peptide, lysozyme, lactoferrin and sPLA

The degree of synergy between CSA-13 and different natural antibacterial agents that are usually present at infection sites against three selected bacterial strains was determined by the checkerboard technique as shown in Table 3. Synergy or partial synergy was observed between CSA-13 and all natural antibacterial agents used in this study when tested against Gram (+) *Staph. aureus* bacteria. In the case of Gram (−) *Ps. aeruginosa* Xen 5 and *H. pylori*, partial synergy was observed with the combination of CSA-13 and LL-37, synergy against *H. pylori* with a combination of CSA-13 and sPLA, and an additive effect against both G (−) bacteria with a combination of CSA-13 and lysozyme. Indifference and partial synergy were observed using CSA-13 with lactoferrin against *Ps. aeruginosa* Xen 5 and *Staph. aureus* ATCC 43300 respectively. In a parallel set of experiments, additive effects and synergy were observed between CSA-13 and sPLA.

Discussion

Topical infections take place within tissue surrounding bacterial entry areas and are usually located on skin and mucosal surfaces. Despite many advances in diagnosis and treatment, these infections are still an important factor associated with patient mortality. Very often topical infections are caused by resistant bacterial strains and, from a local starting point, may propagate to systemic infection, especially in patients with diabetes mellitus, burn trauma or HIV (Gould *et al.* 2007; Patel *et al.* 2008). Furthermore, microbial biofilms are increasingly recognized as important contributors to human disease (Lynch and Robertson 2008). New strategies to prevent and treat these infections, including those associated with biofilms, are needed. One limitation of CAPs and their cationic lipid steroid derivative mimics is nonspecific plasma membrane toxicity that may result in host cell damage. Accordingly, a previous study shows that CSA-13 action at a concentration above *c.* 50 μmol l^{-1} can result in haemoglobin and LDH release indicative of host cell membrane damage. We found that RBC membrane disruption but not bacterial killing activity of CSA-13 was significantly decreased by pluronic F-127. This finding may provide a solution to control toxicity of CSA-13 and potentially other cationic peptide mimics by co-formulation with F-127. Although the presence of pluronic F-127 does not increase CSA-13 antibacterial activity directly, it might enable the use of CSA-13 at a higher concentration. In this study, we also provide experimental evidence that CSA-13 has potential for treatment of topical bacterial infection. In agreement with previous reports (Chin *et al.* 2007), we have shown that CSA-13 kills various clinical isolates of *Staph. aureus* including MRSA strains and *Ps. aeruginosa* within cystic fibrosis sputum. In addition to already published information related to activity of this molecule at infection sites, such as CSA-13 resistance to inactivation by anionic polyelectrolytes (Bucki *et al.* 2007a), or protease (Leszczynska *et al.* 2009) and synergism with exogenous antibiotics (Chin *et al.* 2008; Saha *et al.* 2008), we found that CSA-13 effectively kills different bacterial strains embedded in biofilm and that its antibacterial activity is potentialized by host-secreted antibacterial agents. To investigate CSA-13 activity against *Ps. aeruginosa* Xen 5 biofilm, we used four different techniques and obtained similar results with all of them. Our biofilm study confirmed that luminescence evaluation of transformed bacteria provides a rapid method for real-time monitoring of biofilms (Kadurugamuwa *et al.* 2003). Additionally, our results are in agreement with a recent study showing that CSA-13 has bactericidal activity against *Ps. aeruginosa* even in mature biofilms (Nagant *et al.* 2010). Over the broad range of sub-MIC concentration, cathelicidin LL-37 peptide, which interferes with bacterial membrane lipid packing, lysozyme, which hydrolyses the cross-linkages between chains of peptidoglycans, lactoferrin, which deprives bacteria of iron, and secretory PLA2, which likely kills bacteria due to its lipolytic activity, all increased the antibacterial activity of CSA-13. These data are in good agreement with findings that a combination of natural host antibacterial molecules

acts in an additive or synergistic, but not antagonistic, manner (Singh *et al.* 2000). Therefore, CSA-13 at the infection site, where the concentration of endogenous antibacterial molecules such as LL-37 increase due to the stimulatory effect of bacterial wall products, may enhance topical host antibacterial defence. In conclusion, we show that CSA-13 has a large spectrum of bactericidal activity against planktonic and biofilm bacterial cells. Given that CSA-13's strong antibacterial activity was reduced in blood plasma, CSA-13 application has greater potential to prevent or treat topical bacterial infection. Pluronic F-127 represents a promising tool to reduce CSA-13 membrane toxicity towards host cells. Future safety, efficacy and pharmacokinetic studies *in vivo* should be performed to correlate our *in vitro* findings.

Acknowledgments

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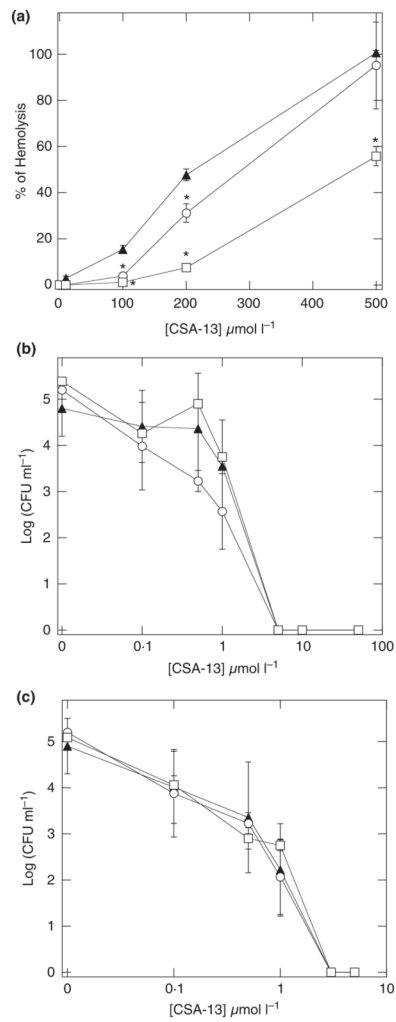
Figure 1.
Haemoglobin release from human red blood cells (panel a) after addition of CSA-13 to their phosphate-buffered saline (PBS) suspension without (▲) or with 1% (○) or 5% (□) of pluronic F-127. Antibacterial activity of CSA-13 against *Pseudomonas aeruginosa* Xen 5 (panel b) and *Staphylococcus aureus* A1 (panel c) in PBS (▲) or PBS containing 1% (○) or 5% (□) pluronic F-127. Error bars represent standard deviations from three measurements. *Significantly different from control sample.
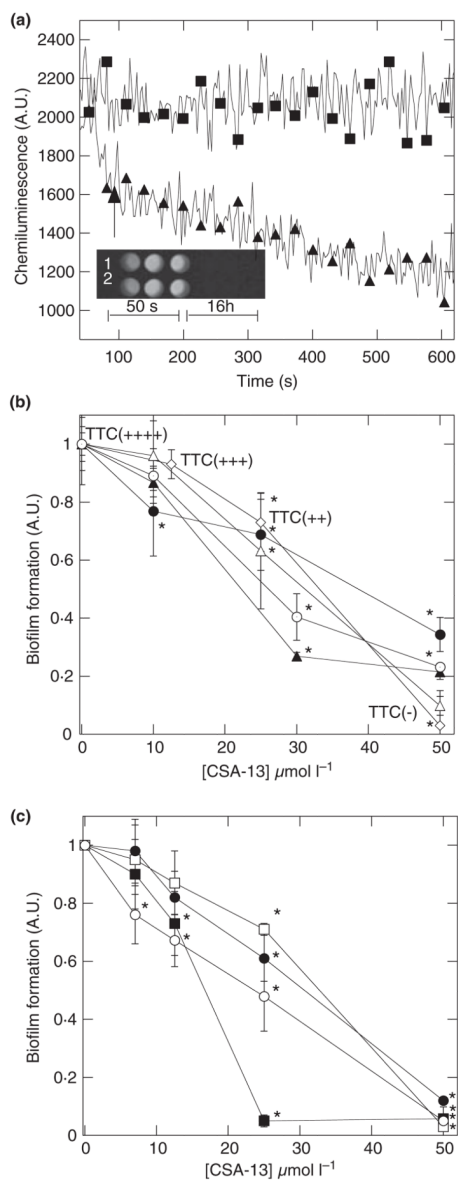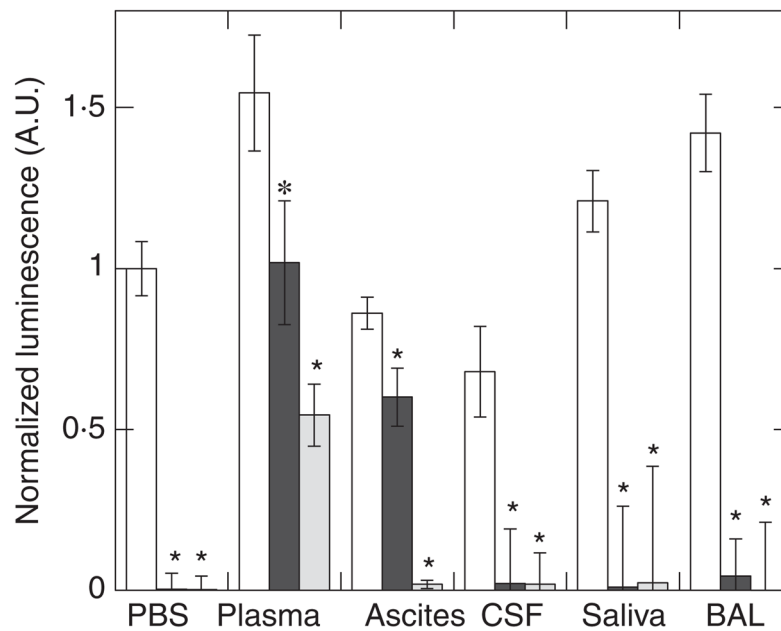
Figure 2.
Bactericidal activity against planktonic and biofilm-forming bacteria. Decrease of
Pseudomonas aeruginosa Xen 5 luminescence (■) after CSA-13 addition (▲) to Tryptic soy
broth (TSB) inoculum with bacterial concentration *c.* 10^8 CFU ml^{-1} (panel a). The insert on
panel a represents the luminescence of *Ps. aeruginosa* Xen 5 (200 μl) at 50 s and 16 h after
CSA-13 (line 1) or polymyxin B (line 2) addition (both at concentration 10 μmol l^{-1}). Data
from one experiment performed in triplicate are shown. Antibacterial activity of CSA-13
against biofilm of *Ps. aeruginosa* Xen 5, evaluated 24 h after its administration (panel b).
Relative value of optical density ($\diamondsuit$), crystal violet absorbance (●), luminescence ($\triangle$),
luminescence in the presence of pluronic F-127 1% (▲) and 5% (☉) at various
concentrations of CSA-13. Antibacterial activity of CSA-13 against biofilm of *Moraxella
catarrhalis* (□), *Helicobacter pylori* (○), *Staphylococcus aureus* ATCC43300 (■) and
Enterococcus faecalis (●), evaluated 24 h after its administration (panel c). On panels b and

c, error bars represent standard deviations from three to five measurements. *Significantly different from control sample.

Figure 3.
Antibacterial activity of CSA-13 against *Pseudomonas aeruginosa* Xen 5 in human body fluids was determined by luminescence readings 6 h after addition of equal amounts of bacteria to an equal volume of phosphate buffered saline (PBS), or PBS supplemented with 50% of plasma, ascites, cerebro-spinal fluid (CSF), saliva or bronchoalveolar lavage (BAL). In each condition, the white column indicates luminescence signal in control samples. Black and grey columns indicate luminescence signal in the presence of 10 and 30 μmol l^{-1} CSA-13 respectively. Data from one experiment performed in triplicate are shown. Two other experiments with samples obtained from different subjects show similar trends. *Significantly different from control sample.

Table 1

Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of CSA-13 against different clinical isolates of *Staphylococcus aureus*

Bacteria source	Strain number	Strain characteristic			CSA-13 (μmol l^{-1})	
		MRSA	MLS$_B$	β-lactamase	MIC	MBC
Surgery wound	1	+	+	+	0·8	3·125
Surgery wound	2	+	+	+	1·56	3·125
Surgery wound	3	+	+	+	0·8	3·125
Diabetic foot ulcer	4	+	+	+	0·8	1·56
Diabetic foot ulcer	5	–	+	+	0·8	3·125
Diabetic foot ulcer	6	–	+	+	1·56	3·125
Diabetic foot ulcer	7	–	+	+	1·56	6·25
Vaginal swabs	8	–	+	+	1·56	6·25
Nasal swab	9	–	–	+	1·56	6·25
Pus from acne	10	–	–	+	1·56	6·25
Pus from acne	11	–	–	+	0·8	3·125
Vaginal swabs	12	–	–	+	0·8	3·125
Nasal swab	13	–	–	–	1·56	6·25
Pus from acne	14	–	–	–	0·8	6·25
Pus from acne	15	–	–	–	0·8	6·25
Pus from furunculus	16	–	–	–	3·125	6·25
Staph. aureus ATCC29213	17	–	–	+	0·8	3·125

Table 2

Antibacterial activity of CSA-13 against clinical strains of *Pseudomonas aeruginosa* [log (CFU ml^{-1})] in the presence of pluronic F-127

Bacteria in CF sputum samples	CSA-13 (μmol l^{-1})	Pluronic (%)		
		0	**1**	**5**
Sample 1				
Ps. aeruginosa (mucoid)	0	5·18	5·20	5·31
Ps. aeruginosa (nonmucoid)	1	4·77	5·14	5·17
MSSA	5	4·74	4·95	4·96
	10	4·86	4·91	5·09
	50	3·16	4·69	4·84
Sample 2				
Ps. aeruginosa (mucoid)	0	3·9	4·12	4·61
Ps. aeruginosa (nonmucoid)	1	4·12	4·35	4·21
MSSA	5	4·05	4·19	4·34
	10	3·92	4·04	4·01
	50	2·17	3·31	3·13
Sample 3				
Ps. aeruginosa (nonmucoid)	0	3·03	2·53	2·25
	1	2·49	2·30	3·22
	5	2·24	2·11	2·21
	10	2·32	3·12	2·74
	50	0	0	0
Sample 4				
Ps. aeruginosa (nonmucoid)	0	4·58	4·76	4·81
	1	4·82	4·86	4·98
	5	4·53	4·80	4·77
	10	4·45	4·35	4·51
	50	2·33	3·74	3·13
Sample 5				
Ps. aeruginosa (nonmucoid)	0	5·56	5·65	5·82
	1	5·58	5·57	5·51
	5	5·56	5·44	5·39
	10	5·49	5·39	5·69
	50	3·22	2·93	4·63
Sample 6				
Ps. aeruginosa (nonmucoid)	0	3·80	4·06	4·09
	1	3·73	3·99	3·74
	5	3·61	3·95	3·69
	10	3·64	3·61	3·78
	50	3·15	3·44	3·47

CF, cystic fibrosis. MSSA, Methicillin sensitive *Staphylococcus aureus.*

Table 3

Interaction between CSA-13 and different host antibacterial agents assessed with fractional inhibitory concentration (FIC) index. FIC$_{index}$ was interpreted as follows: <0·5 (synergy; S), 0·5–0·75 (partial synergy; PS), 0·76–1·0 (additive effect; AE), >1·0–4·0 (indifference; I), >4·0 (antagonism, A)

| Bacterial strain | FIC | CSA-13 with | | | | |
		1 LL-37	2 lysozyme	3 lactoferrin	4 sPLA	Interpretation 1/2/3/4
Pseudomonas aeruginosa Xen 5	FIC$_A$/FIC$_B$	0·368/0·308	0·387/0·572	0·574/0·574	0·472/0·449	PS/AE/I/AE
	FIC index	0·676	0·959	1·148	0·921	
Staphylococcus aureus ATCC 43300	FIC$_A$/FIC$_B$	0·1/0·163	0·104/0·305	0·328/0·515	0·238/0·469	S/S/PS/PS
	FIC index	0·263	0·409	0·843	0·707	
Helicobacter pylori	FIC$_A$/FIC$_B$	0·465/0·168	0·48/0·371	0·371/0·379	0·304/0·093	PS/AE/PS/S
	FIC index	0·633	0·851	0·750	0·397	