Celal Bayar University Journal of Science
Volume 14, Issue 2, p 223-228

Antimicrobial Activity of Phytosphingosine Nanoemulsions against Bacteria and Yeasts

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Received: 8 March 2018
Accepted: 15 May 2018
DOI: 10.18466/cbayarfbe.403152

Abstract

Phytosphingosine (PS) is a natural antimicrobial ingredient present in the mammalian stratum corneum of the skin. The skin surface is the first line defense system against microbial colonization and infections. The aim of this study was to investigate *in vitro* antimicrobial effects of PS and PS-hydrochloride (PSHCl) nanoemulsion (NE) formulations and solutions against bacteria and fungi. Minimum inhibitory concentrations (MICs) of the PS-NE and PSHCl-NE formulations and PS/PSHCl solutions were determined by microdilution method against Gram-positive/Gram-negative bacteria and yeasts. Inhibitory effects were also examined by disc diffusion method and inhibition zone diameters were determined. PS/PSHCl solutions and PS-NE/PSHCl-NE formulations were prepared in different conditions. Physicochemical properties of the NEs were characterized and antimicrobial activities were evaluated. In this study we reported that the PS and PSHCl and NEs had variable antimicrobial activities for a variety of Gram-positive bacteria (MIC range 1 µg/ml to 1024 µg/ml), Gram-negative bacteria (MIC range 8 µg/ml to 1024 µg/ml) and Candida strains (MIC range 8 µg/ml to 512 µg/ml). Sphingolipids are part of the natural defense system of the body. We demonstrated that PS and PSHCl solutions and NEs have broad antimicrobial activity against bacteria and yeasts. In addition, the newly developed formulations may have potential for prophylactic and therapeutic intervention in infections.

Keywords: Phytosphingosine, Phytosphingosine hydrochloride, Nanoemulsions, Antimicrobial activity.

1. Introduction

The human skin, with a surface of approximately 1.6-2 m^2, is very resistant against external threats and microorganisms. The main barrier of the skin, is the stratum corneum. Essential compounds of the stratum corneum are lipids, which are enzymatically transformed to intracellular membrane lipid bilayers, consisting of ceramides, cholesterol, fatty acids and trigiycerides, among others. The complex epidermal sphingolipids ceramides are consisting of a sphingoid base, covalently attached by its amine group to a fatty acid of varying chain length by an N-acyl bond. Phytosphingosine (PS) is one representative of sphingoid bases, in addition to sphinganine, sphingosine and 6-OH-4-sphingenine. Stratum corneum lipids, either free or covalent bound, and its components like PS play a crucial role in the defense mechanism of the skin by increasing its barrier function [1,2]. Due to this fact the question arises if these lipids are capable for more.

PS influences a broad variety of cellular functions, like inflammation of the skin, and increases ceramide levels in and the barrier function of the stratum corneum [3, 4]. Although the composition, biosynthesis, secretion, and function of cutaneous lipids in the skin are well characterized, only little is known about their role in the defense against microbial infections. PS is known to have antibacterial activity and plays more direct role than previously thought in innate immune defense against epidermal and mucosal bacterial infections [5].

Nanoemulsions (NEs) are heterogeneous systems consisting of two immiscible liquids. NEs are emulsions with droplet size under 1 micrometer, typically containing oil, water and an emulsifier. NEs have substantially different physicochemical properties than larger microscopic systems due to their submicron particle diameters [6]. Properties like particle–particle interactions or interaction with biological tissue, among others, are affected by the small particle diameters.

The antimicrobial properties of PS were investigated in a study [7]. However, regarding the literature according to antimicrobial investigations of PS there are two major drawbacks: In general, only skin essential micro-organisms were investigated, and PS was used in

inadequate forms like a chloroform-methanol solution [8] or as an excipient [9, 10]. It would be more appropriate to use PS as an active in formulations suitable for the end use, like NEs. Due to our previous experiences with NEs, PS-NE and PS-HCl-NE were prepared and their antimicrobial activity against bacteria and yeasts was investigated.

2. Materials and Methods

2.1. Materials

PS and PS-HCl were kindly gifted by Evonic Industries (Germany), oleic acid was from Sigma Aldrich, St. Louis, MO, Tween 80 and ethanol (99,9%) were from Merck (Germany), Lecithin (90% soybean phosphatidyl ethanolamine) was from Applichem GmbH (Germany). Dimethyl sulfoxide (DMSO) and water (1:1) were used for the dissolution of PS and PSHCl. American type culture collection (ATCC) and Refik Saydam culture collection (RSKK) strains were used. Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212 and *Bacillus subtilis* RSKK 02021), Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Salmonella enterica* RSKK 04059) and yeasts (*Candida albicans* ATCC 90028 and *Candida parapsilosis* RSKK 04057) were tested. The strains were stored at -80°C in Brain Heart infusion broth (Merck, Germany) with 10% glycerin before antimicrobial activity experiments.

2.2. Methods

2.2.1. Preparation of the nanoemulsions

The PS and PCHCl containing NEs and solutions (Table 1) were produced as follows: The PS solution was prepared by dissolving 1 % (w/w) PS in oleic acid. The PSHCl solution was prepared by dissolving PSHCl in a mixture of water and ethanol (75+25) (w/w) at room temperature.

The NEs were prepared by using a high speed stirrer: The water phase and oliy phase were prepared separately. The oil phase (40 %) of the PS-NE was obtained by dissolving 1 % PS and 2 % Lecithin in oleic acid (w/w). The water (60 %) was prepared by dissolving 2 % Tween 80 in distilled water (w/w). The water phase was added to the oil phase and stirred by using the high speed stirrer Ultra-Turrax T8 (IKA) with 8000 rpm for 3 min at room temperature. The oil phase (60 %) of the PSHCl-NE was obtained by solving 2 % Lecithin in oleic acid. The water phase (40 %) was prepared by dissolving 1 % PSHCl and 1 % Tween 80 in a mixture of water and ethanol (15+25) (w/w) at room temperature. The water phase was added to the oil phase and stirred by using the high speed stirrer Ultra-Turrax T8 (IKA) with 8000 rpm for 3 min at room temperature.

Table 1. Ingredients of PS and PSHCl solutions and NEs (all values as weight).

	PS solution	PS NE	PSHCl solution	PSHCl NE
oil phase			-	
oleic acid	9.9	3.7	-	5.8
PS	0.1	0.1	-	-
Lecithin	-	0.2	-	0.2
water phase	-			
PSHCl	-	-	0.1	0.1
Tween 80	-	0.2	-	0.1
Etanol	-	-	2.5	2.5
purified water	-	5.8	7.5	1.5

2.2.1.1. Characterization

The PS NEs were characterized in terms of particle size, particle size distribution and zeta potential (ZP), while the ZP measurement for the PSHCl NE was not performed, because it is not meaningful. Particle size and ZP were measured with Zetasizer NanoZS (Malvern, USA) using the non-invasive back scattering (NIBS) technique and laser doppler microelectrophoresis technique, respectively. Size measurements were performed in disposable polystyrene microcuvettes, and zeta potential measurements were carried out in standard zeta cuvettes. Samples were measured after 30-fold dilution with ultrapure water.

2.2.3. Antimicrobial activity

Inhibitory effects of PS and PSHCl NEs and solutions were examined by disk diffusion and broth microdilution methods.

2.2.3.1. Disk diffusion method

Inhibition zone diameters of the formulations and the solutions on different bacteria and fungi were performed,

as previously described, by disk diffusion method with minor modification [11]. Bacteria were grown on Mueller-Hinton agar (MHA) (Merck, Germany) and *Candida* strains were grown on Sabouraud dextrose agar (SDA) (Oxoid, UK) for 24 h at 35°C. Fresh microbial colonies were suspended in physiological saline and inoculum suspensions were adjusted to 0.5 McFarland turbidity with densitometer device (Biosan, Latvia). The surface of the MHA and SDA plates was covered with the suspentions using sterile cotton swabs and the plates were air dried for 15 minutes before the sterile blank disks (6 mm in diameter) (Oxoid, UK) were laid on the surface of the inoculated plates. 10 µL of each solution and formulation were added onto the sterile disks and the plates were incubated at 35°C for 16-20 h. The inhibition zones were measured in milimetres. Gentamicin and fluconazole were used as references and the quality control ranges were evaluated according to Clinical and Laboratory Standards Institute (CLSI) and The European Committee on Antimicrobial Susceptibility Testing (EUCAST). Each sample was tested in triplicate and the mean zone diameters ± standard deviations (SD) were reported.

2.2.3.2. Broth microdilution method

Minimum inhibitory concentrations of the formulations and the solutions were determined by broth microdilution method against bacteria and yeasts [11]. Bacterial and fungal strains were grown for 24 h at 35°C on MHA and SDA, respectively. The fresh colonies of bacteria and fungi were suspended in physiological saline solution, the cell density was adjusted as described above and the suspensions were diluted 100 fold. 50 µl of cation adjusted MHB were pipetted in each well of a 96well plate for bacteria. 50 µL of RPMI medium (Sigma, Germany), buffered with 3-(N-morpholino) propanesulfonic acid were pipetted in each well of a 96-well plate for yeasts. 50 µL of the solutions and the formulations were added into the first wells and serial dilutions were performed. Finally 50 µL suspension of each bacteria or yeasts were added to the wells and incubated overnight at 35°C. The lowest concentration that inhibited the growth of the microorganism was defined as MIC value. Each sample was tested in triplicate. Ciprofloxacin and fluconazole were used as reference agents for antimicrobial activity and the quality control ranges were evaluated according to CLSI and EUCAST.

3. Results and Discussion

The mean droplet size of the PS-NE was 411 nm, with a polydispersity index (PDI) of 0.56 and a zeta potential (ZP) of 28 mV (Table 2). The amount of oleic acid in the PSHCl-NE was 60 %, the prepared formulation was w/o. Due to this fact that the measurement of ZP is not meaningful and was not performed. The mean droplet size of the PSHClNE was much higher, in the micrometer range.

Table 2. The mean droplet size, polydispersity index and zeta potentials of the NEs.

	PS-NE				**PSHCl-NE**	
	Z-Ave [nm]	PDI	ZP [mV]		Z-Ave [nm]	PDI
	448	0,50	28,2		>1 µm	0,53
	432	0,50	27,4		>1 µm	1
	353	0,68	27,4		>1 µm	0,10
mean	411	0,56	27,7	mean	>1 µm	0,54

PS, PSHCl solutions and NEs of these substances displayed antimicrobial activity against a variety of Gram-positive bacteria, Gram-negative bacteria and yeasts. The results of disk diffusion and microdilution methods showed that PS and PSHCl (both solutions and NEs) are potent agents for fighting against some microorganisms. PS, PSHCl and NEs were active against three Gram-positive bacteria (S. aureus, E. faecalis and B. subtilis) and two yeasts (C. albicans and C. parapsilosis) tested. On Gram-negative bacteria they were effective against two of three tested strains (*E.coli* and *S. enterica*).

The mean inhibition zone diameters against different species of bacteria and yeasts are shown in Table 3. Disk diffusion test performed with the PSHCl against microorganisms generated wider inhibition zones than PS. The NEs and the solutions of PS were found to have no inhibitory effects on E. faecalis, C. albicans and C. parapsilosis strains at studied concentrations. PS and PSHCl were also ineffective against P. aeruginosa at studied concentrations. Larger zone diameters were observed in the presence of PSHCl/PSHCl-NE as compared to PS/PS-NE. The inhibition zone diameters of the NEs and the solutions for the strains were almost similar to each other when compared in pairs.

Table 3. Inhibition zone diameters (mm) for bacteria and yeasts (Mean zone diameter ± SD).

	NEs		Solutions		Control	
Strains	**1% PS**	**%1 PSHCl**	**1% PS**	**1% PSHCl**	**Blank NE**	**DMSO**
S. aureus	12±0	14,66±0,57	11,66±0,57	16±0	-	-
E.faecalis	-	15±0	-	16±0	-	-
E. coli	10±0	12±0	11±0	13±0	-	-
S. enterica	9±0	12,66±0,57	11±0	13±0	-	-
P. aeruginosa	-	-	-	-	-	-
B. subtilis	12±0	16±0	11±0	16±0	-	-
C. albicans	-	8±0	-	9,33±0,57	-	-
C. parapsilosis	-	9±0	-	9±0	-	-

*(-): no inhibition zone

Minimum inhibitory concentrations of the formulations and the solutions on bacteria and yeasts are presented in Table 4. MIC of PS-NE was determined as 4 µg/ml and 8 µg/ml against *E. faecalis* and *B. subtilis*, respectively. MICs of PS-NE and PS solution were equal to 256 µg/ml and 512 µg/ml against *C. parapsilosis* and *C. albicans*, respectively. In the presence of PS-NEs, MIC levels decreased more than 16-fold against *S. aureus*, *E. faecalis* and *B. subtilis* as compared to PS solution.

In the presence of PSHCl-NE the MIC was determined as 1 µg/ml against *E. faecalis*, 2 µg/ml against *B. subtilis* and 4 µg/ml against *S. aureus*. In the presence of PSHCl solution the MIC was 8 µg/ml against *E. coli., S. enterica, C. albicans* and *C. parapsilosis.* PSHCl solution was also effective against *S. aureus*, *E. faecalis* and *B. subtilis* with MIC of 1 µg/ml. Inhibitory activity of PSHCl solution was two-times greater against *S. aureus*, *E. coli, S. enterica* and *B. subtilis* as compared to PSHCl-NEs.

Table 4. Minimum inhibitory concentrations (µg/ml) of the formulations.

Strains	NEs		Solutions		Control	
	1% PS	**%1 PSHCl**	**1% PS**	**1% PSHCl**	**Blank NE**	**DMSO**
S. aureus	32	4	512	1	-	-
E.faecalis	4	1	1024	1	-	-
E. coli	1024	64	1024	8	-	-
S. enterica	1024	32	1024	8	-	-
P. aeruginosa	>1024	>1024	>1024	>1024	-	-
B. subtilis	8	2	512	1	-	-
C. albicans	512	64	512	8	-	-
C. parapsilosis	256	64	256	8	-	-

*(-): no effect

Sphingoid bases, neutral lipids and fatty acids are involved in the stratum corneum and contribute to the physical and permeability barrier and to the immune defense of the skin. Although the antimicrobial activities of these common lipids against both Gram-positive/Gram-negative bacteria and fungi have been established, little is known about the mechanisms of action and their role in microbial infections and colonization [8, 12, 13]. Antibacterial lipids like free sphingosines, dihydrosphingosines, lauric acid and sapienic acid have been shown to be present in the oral cavity, in saliva and at mucosal surfaces [8]. Sphingolipids like PS are involved at high levels in the mammalian stratum corneum and in the immunologic barrier functions of the skin [1, 2] and the composition, biosynthesis, secretion and functions of these lipids are well characterized. Certain lipids, peptides, stratum corneum integrity, dry surface, low pH and products of endogenous nonpathogenic bacteria contribute to the inhospitable environment for pathogenic microorganisms and the lipids may be the major contributors to this situation [13]. In addition to the antimicrobial effects of the lipids alone, antimicrobial lipids and antimicrobial peptides can act synergistically against microorganisms [12, 14].

PS is known to have antibacterial activity and plays more direct role in innate immune defense against epidermal and mucosal bacterial infections [8]. PS influences a broad variety of cellular functions, like inflammation of the skin, increasing ceramide levels in the barrier function of the stratum corneum [3, 4].

The antimicrobial, anti-biofilm and antiinflammatory properties of sphingolipids and its derivatives were investigated in several studies [7, 15]. Sphingosine, dihydrosphingosine and PS were shown to be similarly active against a range of Gram-positive and Gram-negative bacteria [12] and the long-chain bases were shown to be ineffective against *P. aeruginosa* or *Serratia marcescens* [9]. Similar to the results of Fischer et al. [8, 12] we demonstrated that PS and PSHCl have potent

inhibitory effects against a range of Gram-positive and Gram-negative bacteria (except for *P. aeruginosa*).

PS has potent antimicrobial activity and low toxicity that may have applications to the novel formulations in order to prevent or treat several skin infections [9 Fischer]. Although there are several types of formulations containing PS in dermatological studies [16, 17], it is necessary to research the antimicrobial effects of the novel formulations in colonization and microbial infections. NEs contain oil, water and an emulsifier and the use of PS and PSHCl NEs would be more appropriate in skin infections. PS and PSHCl may contribute to defensive barrier of the skin and have potential for prophylactic intervention and microbial infections.

Although little is known about the exact mechanism of antimicrobial activity of sphingolipids, there are several possibilities. Lipids may penetrate and disrupt the cell wall of the bacteria; they may alter the cytoplasmic membrane of the microorganism and it is also possible that the lipids may directly penetrate the cell walls and cytoplasmic membranes of bacteria; they may enter and disrupt the cytoplasm [8, 18, 19].

In this study, we showed that the PS and PSHCl and their NEs had variable antimicrobial activities for a variety of Gram-positive bacteria (MIC range 1 µg/ml to 1024 µg/ml), Gram-negative bacteria (MIC range 8 µg/ml to 1024 µg/ml) and Candida strains (MIC range 8 µg/ml to 512 µg/ml). Pavicic et al. [15] showed clinical results in the context of skin care in acne, based on both anti-inflammatory and antimicrobial activity. Pavicic et al. demonstrated that PS inhibited the growth of Gram-positive and Gram-negative bacteria and yeasts at very low concentrations in parallel with our study. The concentrations required for growth inhibition (within 1 h) were 0.0012% for *C. albicans* and 0.04% for *E. coli* [15]. In a previous study, Bibel et al. [7] showed that sphingosines caused a significant reduction in colony forming units of *S. aureus* (4.5 log) at a concentration of 6.5 µg/ml. In another study, sphingosines (and its isomers) and stearylamine were also found to be effective against *S. aureus, Streptococcus pyogenes*, *Micrococcus luteus*, *Proprionibacterium acnes*, *Brevibacterium epidermidis* and *C. albicans* [7]. Sphingosine-1phosphate was shown to have antimicrobial activity against *Mycobacterium* species [20]. Sphingosine, dihydrosphingosine and PS were active against a range of bacteria including *S. aureus, E. coli*, *Fusobacterium nucleatum*, Streptococci and *Corynebacterium* species and minimum bactericidal concentrations were in the range of 0.3-63 µg/ml [8, 12]. In this study the inhibitory concentrations of PS and PSHCl were variable and depended upon the microorganism and the formulation's nature (solution or NE). The results of this study confirm the previous results on potent antimicrobial effectiveness of sphingosines.

4. Conclusion

Sphingoid bases are involved in the stratum corneum and contribute to the immunologic functions of the skin by several mechanisms. It is necessary to identify new antimicrobial compounds because of the increasing rates of resistant pathogens. The data obtained from this study highlight the potential of using PS and PSHCl as alternative antimicrobials against pathogenic bacteria and yeasts. Novel formulations like NEs including PS and PSHCl as active compounds may have potential for prophylactic intervention and may provide for new therapeutic opportunities against infections and microbial colonization.

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