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Decade-long use of the antimicrobial peptide combination tyrothricin does not pose a major risk of acquired resistance with gram-positive bacteria and *Candida* spp

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Tyrothricin, an antimicrobial peptide combination produced by *Bacillus brevis* consisting of gramicidins and tyrocidins commands broad antimicrobial activity against gram-positive bacteria and some yeasts *in vitro*. The polypeptide and its components have been used therapeutically for about 60 years in the local treatment of infected skin and infected oro-pharyngeal mucous membranes. Though older studies suggest that resistance development of originally susceptible microorganisms towards tyrothricin is a rare event, data concerning recent state of resistance are lacking. In the present *in vitro* study the susceptibility to tyrothricin of clinical isolates of bacterial and yeast origin from superficial swabs of the skin and mucous membranes of outpatients and inpatients obtained from clinical material in the second half of the year 2003 was determined. Using a microdilution assay, the minimum inhibitory concentration (MIC and MIC_{90}, defined as the concentration that inhibits at least 90 percent of the tested strains) of 20 strains each of *Staphylococcus aureus* of the variety MSSA (susceptible to methicillin), *Staphylococcus aureus* of the variety MRSA (methicillin resistant), *Staphylococcus haemolyticus*, *Streptococcus pyogenes, Enterococcus faecalis, Corynebacterium* spec., *Candida albicans* and *Candida parapsilosis* was determined. All of the tested gram-positive bacteria turned out to be highly susceptible to tyrothricin with MICs $\leq$ 4 mg/l. The tested yeast strains were susceptible to the polypeptide antibiotic as well, but (with MICs of 16 mg/l and 32 mg/l, respectively) to a lesser extent. No acquired resistance of the tested strains was determined, indicating that the risk of resistance development against topically applied tyrothricin is only marginal, if there is any at all. Thus, long term-, i.e. decade-long use of topically applied tyrothricin and its components in the local treatment of infected skin does not pose a major risk with respect to acquired resistance of originally susceptible gram-positive bacteria and yeasts, not even in the case of *Staphylococcus aureus,* both with MSSA and MRSA strains. The broad anti-bacterial and anti-fungal activity of tyrothricin combined with its lacking risk for resistance development make the antimicrobial peptide a valuable addition to our therapeutic armamentarium in the treatment of infected skin.

1. Introduction

Tyrothricin is an antimicrobial peptide combination produced by *Bacillus brevis* (Dubos 1939) consisting of linear and cyclic polypeptides (70-80 % tyrocidins, 20-25 % gramicidins) (Vogt 1989). Both peptides command broad antibacterial activity against gram-positive cocci and rods (Schneider 2005) being the result of intercalation of the peptides into bacterial membranes. This leads to the formation of hydrophilic ion channels rapidly causing cell leakage and cell death (Franklin and Snow 1988; Seoh and Busath 1993). Besides its antibacterial activity, tyrothricin is also able to inactivate viruses like Sendai virus (Grossgebauer and Hartmann 1978), herpes simplex virus (Grossgebauer and Hartmann 1979) and HIV (Human immunodeficiency virus) (Bourinbaiar 1994). Because of its toxicity towards eukaryotic cells (Muramatsu et al. 1972), the use of tyrothricin as antibiotic is restricted to local application (Willenberg 1979) in particular for the treatment of infected skin and infected oro-pharyngeal mucous membranes.

In the context it has been shown that tyrothricin *in vitro* is highly active against gram-positive aerobic bacteria isolated from the normal flora of the respiratory tract with minimal inhibitory concentrations (MICs) ranging from 0.046 mg/l (for *Streptococcus pyogenes*) to 3.12 mg/l (for *Staphylococcus aureus*) (Kretschmar et al. 1995). An *in vitro* antibacterial activity of tyrothricin could also be demonstrated against streptococci including enterococci (Kretschmar et al. 1995; Ruckdeschel 1983), yeasts like *Candida albicans* (Kretschmar et al. 1996b) and - particularly noteworthy - methicillin resistant strains of *Staphylococcus aureus* (MRSA) (Kretschmar et al. 1995, 1996b).

Older studies point out that tyrothricin and its component gramicidin themselves do not seem to propagate the development of antibiotic-resistant organisms (Ruckdeschel 1983; Lind 1983). Generally, resistance development to tyrothricin seems to be a

rare event in these studies (Ruckdeschel 1983). For example, the prolonged use of tyrothricin tooth paste over 2 years by 99 subjects neither led to an increase in the numbers of tyrothricin-resistant microorganisms nor to an increase in cross resistance to penicillin, streptomycin, tetracyclines and chloramphenicol (Lind 1983). However, data regarding recent state of resistance are lacking although these peptides have been therapeutically used for about 60 years by now.

2. Investigations and results

In the present *in vitro* study the susceptibility to tyrothricin of clinical isolates from superficial swabs of the skin and mucous membranes of outpatients and inpatients obtained from clinical material in the second half of the year 2003 was determined.

Aim of the investigation was to ascertain the actual MICs of bacterial and yeast strains from the human skin and mucosa compared to reference strains such as *Staphylococcus aureus* MSSA*, Enterococcus faecalis and Candida albicans* that are known to be susceptible to tyrothricin.

Furthermore, the obtained MICs should be checked against former data from the literature concerning this topic. Finally, it was tried to quantify the acquired resistance of the isolated strains according to the "Guideline on the Pharmacodynamic Section of the SPC for anti-bacterial Medicinal Products" (CHMP/EWP/520/96)".

The results of MIC determination are shown in Table 1. Tyrothricin inhibited all the analysed gram-positive cocci including staphylococci, streptococci, and enterococci. In detail, all of the determined 20 strains of *Staphylococcus aureus* MSSA (MIC 4 mg/l), *Staphylococcus aureus* MRSA (MIC 4 mg/l), *Staphylococcus haemolyticus* (MIC 4 mg/l), *Streptococcus pyogenes (MIC 0.5 mg/l)*, and *Enterococcus faecalis* (MIC 2 mg/l; 1 individual strain 8 mg/l) turned out to be highly susceptible against tyrothricin with MICs $\leq$ 4 mg/l.

Moreover, gram-positive rods like *Corynebacterium* spec. proved to be highly susceptible to tyrothricin (MIC 2 mg/l). All of the tested yeast strains of *Candida albicans* and *Candida parapsilosis* were usceptible to the polypeptide antibiotic as well, but (with MICs of 16 mg/l and 32 mg/l, respectively) to a lesser extent compared to the gram-positive bacteria.

As expected, the reference strains *Staphylococcus aureus* MSSA ATCC 29213, *Enterococcus faecalis* ATTCC 29212, and *Candida albicans* (ATCC 10231) were also sensitive to tyrothricin (MICs of 2 mg/l, 2 mg/l, and 4 mg/l, respectively) whereas the strains of the growth control, i.e. 5 strains each of *Acinetobacter baumannii, Enterobacter cloacae, Enterobacter agglomerans, Proteus mirabilis*, and *Pseudomonas aeroginosa*, showed a natural resistance against the polypeptide antibiotic with MICs > 1024 mg/l, respectively (individual data not shown).

It was not possible to determine any acquired resistance of the tested strains according to the "Guideline on the Pharmacodynamic Section of the SPC for anti-bacterial Medicinal Products" (CHMP/EWP/520/96) because none of the tested microorganisms, neither bacteria, nor yeasts, showed any growth under the influence of tyrothricin at a concentration $\geq$ 100 mg/l – apart from the growth controls.

3. Discussion

Antimicrobial peptides, also known as host defense peptides, have been identified in various species ranging from bacteria to mammals, including humans (Wang and Wang 2004). Until now, more than 1200 antimicrobial peptides from different origins bacteria, plants, insects, reptiles and mammals have been reported (Wang et al. 2009). Generally, antimicrobial peptides are small, cationic, amphiphilic peptides with broad-spectrum microbicidal activity against both bacteria and fungi (Schneider 2005). In mammals, they form the first line of host defense against pathogenic infections and generally play an important role as effector molecules of the innate immune system (Steinstraesser 2008) whereas the special function of the peptides in bacteria is still unknown.

Most antimicrobial peptides possess 12 to 50 amino acid residues with net positive charges of + 2 or more and display both cationic and hydrophobic surfaces (Schneider et al. 2005). It is generally accepted that these cationic peptides selectively interact with anionic bacterial membranes (Schneider 2005; Khandelia 2008) and that their positive charge is a prerequisite for this ability (Schneider 2005) Currently, there is great interest in antimicrobial peptides because these 'nature's antibiotics' are considered as promising agents for virtually new therapeutic approaches in infectious diseases especially of the skin and for wound healing (Steinstraesser 2008). Furthermore, antimicrobial peptides might contribute to overcome the growing problems of antibiotic resistance (Wang and Wang 2004; Hancock 2002; Bradshaw 2003) in the treatment of these diseases due to their special mode of action against microorganisms, namely directly targeting and destroying their membranes.

The mentioned mode of action has also been described for the antimicrobial peptide tyrothricin which is known to form hydrophilic ion channels in membranes of microorganisms rapidly causing cell leakage and cell death (Franklin 1988; Seoh 1993). Because of its toxicity towards eukaryotic cells (Muramatsu et al. 1972) tyrothricin should only be applied locally (Willenberg 1979), in particular for the treatment of infected skin and infected oro-pharyngeal mucous membranes. Since tyrothricin or its component gramicidin have been in therapeutical use for about 60 years (Ruckdeschel 1983) and have demonstrated their efficacy in older clinical studies (Lammers 1983; Florestano et al. 1956) without any observable resistance development (Lind 1983), it is of great interest if recent susceptibility of formerly sensitive gram-positive bacterial and yeast species towards the antimicrobial peptide is as pronounced as in older investigations and thus could be preserved over the years.

With this *in vitro* study it could be demonstrated that all the analysed gram-positive bacteria including staphylococci, streptococci, and enterococci as well as Corynebacterium spec. and yeast species, namely *Candida albicans* and *Candida parapsilosis,* still turn out to be highly susceptible to tyrothricin (see Table 1). The MICs ascertained in this study confirm respective data concerning the spectrum of activity and antibacterial/antifungal effect of tyrothricin from the nineties (Kretschmar 1995, 1996a, 1996b; see also Table 2) and even surpass susceptibility data from the eighties in which, for example, ranges of inhibition of 2 to 256 μg/ml were found for both *Corynebacterium* spec. and *Staphylococcus* spec. (Ruckdeschel 1983). Thus, in the experiments presented here it was not possible to find any indication of the development of acquired resistance towards the antimicrobial peptide (not even in *Staphylococcus aureus* MSSA and MRSA strains) indicating that the risk of resistance development against topically applied tyrothricin is negligible.

The data are in accordance with clinical data indicating that the topical use of tyrothricin in the treatment of skin diseases is still effective. In detail, the efficacy and tolerability of an antiseptic wound powder based on tyrothricin (trade name: Tyrosur® Powder, manufacturer: Engelhard Arzneimittel GmbH & Co KG, Niederdorfelden, Germany) was demonstrated in a prospective, randomized multi-centre trial with 131 patients with posttraumatic and surgical cutaneous lesions. A superior efficacy of tyrothricin powder compared to placebo powder

Table 1: MIC_{s90} (mg/l) of tyrothricin for 20 isolated strains each of gram-positive bacteria and yeasts (S.: *Staphylococcus*; Str.: *Streptococcus*). RFS indicates the respective reference strain from ATCC.

S. aureus MSSA	*S. aureus* MRSA	*S. haemolyticus*	*Str. pyogenes*	*Enterococ. faecalis*	*Corynebact. spec.*	*Candida albicans*	*Candida parapsilosis*
2	2	2	0.25	8	1	8	32
1	4	2	0.5	2	2	8	8
2	2	1	0.5	2	1	8	16
4	2	2	0.5	2	2	8	32
4	2	4	1	2	1	8	16
2	2	2	0.5	2	2	8	8
4	2	2	0.5	2	1	8	8
4	2	2	0.25	2	0.5	8	8
2	2	2	0.5	2	1	16	8
8	4	2	0.5	2	2	16	4
2	2	1	0.5	2	2	16	8
2	2	1	0.5	2	1	16	8
4	2	2	0.25	2	0.5	4	16
4	2	4	0.5	2	2	8	16
4	1	4	0.5	2	1	4	8
2	2	2	0.5	2	1	4	8
4	2	2	0.5	2	1	8	32
2	2	2	0.5	2	2	8	16
2	2	2	0.5	1	1	8	16
2	8	2	0.5	2	1	8	8
2 (RFS)				2 (RFS)		4 (RFS)	

Table 2: MIC_{s90} of tyrothricin for selected bacterial and yeast strains determined in the present *in vitro* study compared to respective data from literature: Kretschmar et al., 1995, 1996a, 1996b

Tested strains	MIC_{90} of tyrothricin (mg/l)	MIC_{90} of tyrothricin (mg/l), data from literature
Staphylococcus aureus MSSA	4	1,56
Staphylococcus aureus MRSA	4	1,6-3,2
Streptococcus pyogenes	0,5	0,1-0,4
Enterococcus faecalis ATCC 29212	2	1,6
Candida albicans	16	4,8-6,7

could be demonstrated relating to the radius of the lesions ($p = 0.016$) as well as the wound index ($p = 0.005$) while the tolerability of verum and placebo treatment were comparable (Bayerl 2004).

These findings together with the results obtained support the assumption that tyrothricin is capable to accelerate wound healing in case of local infection or danger of infection. With its broad anti-bacterial and anti-fungal activity combined with the finding of a lacking risk for resistance development in a long term use of topical application tyrothricin and its components it may represent a valuable addition to our therapeutic armamentarium in the local treatment of infected skin – based on our current state of knowledge without any risk of resistance development.

4. Experimental

4.1. Drug

Tyrothricin obtained from Engelhard Arzneimittel GmbH & Co KG, Niederdorfelden, Germany, was dissolved in methanol at a concentration of 16.384 g/l and further diluted 1:2 with Aqua bidest (B. Braun-Melsungen AG, Melsungen, Germany) and then 1:4 with test medium (H broth for analyzing *Streptococcus pyogenes* and *Corynebacterium* spec. and Iso-Sensitest broth for analyzing other strains, both from Merlin Diagnostika, Bornheim-Hersel, Germany). The final concentration of tyrothricin in the achieved solution amounted to 2048 mg/l. For conducting the microdilution assays, the tyrothricin solution was further diluted with the respective test medium.

4.2. Microorganisms

Clinical isolates were obtained from clinical material sent to the laboratory in the second half of the year 2003. The material originated from superficial swabs of the skin and mucous membranes of outpatients and inpatients. Bacterial and yeasts strains were isolated and characterized using standard methods (Murray 2003) and stored in the Mast Cryobank (Mast Diagnostica GmbH, Reinfeld, Germany) at -20 °C before use. Within the study, 20 strains each of *Staphylococcus aureus* MSSA, *Staphylococcus aureus* MRSA, *Staphylococcus haemolyticus*, *Streptococcus pyogenes, Enterococcus faecalis, Corynebacterium* spec., and *Candida albicans* as well as *Candida parapsilosis* were investigated. Reference strains were obtained from the American type culture collection (ATCC): *Staphylococcus aureus* MSSA ATCC 29213, *Enterococcus faecalis* ATTCC 29212, and *Candida albicans* (ATCC 10231). Five strains each of *Acinetobacter baumannii, Enterobacter cloacae, Enterobacter agglomerans, Proteus mirabilis,* and *Pseudomonas aeruginosa* (all of them clinical isolates) known to show a natural resistance to tyrothricin were also used as references.

4.3. In vitro MIC determination

The antibacterial and antifungal activities reflected by the minimum inhibitory concentrations (MICs as well as MICs90, defined as the concentration that inhibits 90 percent of the tested strains) of the bacterial and yeast strains, respectively, were determined using a microdilution method according to DIN 58940. Thawed isolated bacterial strains and reference strains were grown for 18 h at 37 °C on tryptose agar (Difco, Augsburg,

Germany) and the thawed yeast cells on Sabouraud glucose agar (Difco, Augsburg, Germany). Material from five colonies of each strain was used for the preparation of a suspension conforming to McFarland 0.5 in sterile NaCl and inoculated into the test medium: In the case of Streptococcus pyogenes and Corynebacterium spec., 200 µl of the adjusted NaCl-solution into 11 ml H broth (Merlin, Bornheim, Germany) and in the case of the other strains, 100 µl into 11 ml of Iso-Sensitest broth (Oxoid, Wesel, Germany). Aliquots of 50 µl of the inoculated test medium were pipetted to 50 µl each of dilutions of tyrothricin solution in a round-bottom microplate (Greiner, Solingen, Germany) so that the final concentration of tyrothricin in the assay ranged from 1024 mg/l to 0.125 mg/l. Five strains each of *Acinetobacter baumannii, Enterobacter cloacae, Enterobacter agglomerans, Proteus mirabilis*, and *Pseudomonas aeruginosa* were used as growth controls since anaerobic bacteria and enterobacteriacea are known to be unsusceptible towards tyrothricin (Ruckdeschel 1983; Baker 1985). The strains of the growth control were exposed to the maximum concentration of tyrothricin (1024 mg/l). After an incubation period of 24 h at 37 °C the growth of the strains and the MIC were analyzed visually provided that the respective growth control was positive.

4.4. Determination of the acquired resistance

The acquired resistance was determined using the breakpoint concept according to the "Guideline on the Pharmacodynamic Section of the SPC for anti-bacterial Medicinal Products" (CHMP/EWP/520/96). However, evidence-based breakpoints for tyrothricin do not exist because the drug is exclusively applied locally. Fortunately, the concentrations of tyrothricin in saliva samples reached after application of lozenges can be used for orientation purposes. After application of those lozenges containing 4 or 10 mg tyrothricin, maximum levels of the polypeptide antibiotic of 14.4 and 109.3 mg/l were determined in the saliva, respectively (Matula 1988) Though data concerning the tyrothricin level in wounds after the local application of tyrothricin gel (0.1 g/100 g) or powder (1000 mg/kg) are lacking we suppose that those levels in wounds at least reach the magnitude of the saliva levels. Therefore we and others (Ruckdeschel 1983) assume that bacterial and yeast strains are considered susceptible to tyrothricin if the determined MIC is < 100 mg/l.

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