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Spermicidal bacteriocins: Lacticin 3147 and subtilosin A

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Abstract—Spermicidal compounds that also exhibit antimicrobial properties would be extremely attractive agents as they could be used to not only prevent unwanted pregnancy but also to combat the growing prevalence of sexually transmitted infections (STI). One class of compounds that are potential candidates for development of dual-acting contraceptive products are antimicrobial peptides (AMPs). Herein, we report preliminary studies carried out to investigate the spermicidal activity of two bacteriocins, lacticin 3147 and subtilosin A, on bovine, horse/pony, boar and rat sperm.

The increase in the transmission of human immunodeficiency virus (HIV) and sexually transmitted infections (STI) has become a global issue. In addition, there are an estimated 133 million unintended pregnancies every year. Presently marketed contraceptive products typically incorporate the membrane surfactant nonoxynol-9 (N-9) as the main spermicidal ingredient. Although this compound causes the immobilization of sperm, it does not protect against STI.[1] Thus, spermicidal products that also combat HIV/STI are in great demand given that condoms are the only available product on the market that are capable of preventing unwanted pregnancy and HIV/STI infections.[2] Antimicrobial peptides (AMPs) may offer a plausible alternative or enhancement to the existing chemical options as spermicidal and microbicidal agents. Most AMPs are cationic, hydrophobic peptides and exert their properties by binding to the anionic phospholipids present on the outer surface of bacterial cell membranes.[3]

In contrast, the anionic phospholipids of mammalian cells are present along the cytoplasmic side of the cell membrane and the outer surface is composed of zwitterionic phospholipids. This explains the low cytotoxicity of many AMPs towards mammalian cells and further implicates AMPs as suitable microbicidal agents.[4] The recent discovery of the spermicidal properties of nisin A,[5,6] a lantibiotic[7] AMP produced by the food-grade organism *Lactococcus lactis* (Fig. 1, **1**), prompted us to evaluate the spermicidal properties of two other bacteriocins, lacticin 3147 (Fig. 1, **2** and **3**) and subtilosin A (Fig. 1, **4**).

Lacticin 3147 is a two-component lantibiotic (consisting of LtnA1 **2** and LtnA2 **3**) produced by *L. lactis*, that binds lipid II and exhibits a similar antimicrobial activity spectrum as nisin A **1**.[8–10] Subtilosin A is an unclassified cyclic bacteriocin produced by *Bacillus subtilis* and contains three unusual sulfur-α-carbon bridges (Fig. 1, **4**).[11,12] The spermicidal activity of lacticin 3147 and subtilosin A has not previously been established and this is the first report demonstrating such properties of these two bacteriocins.

Bacteriocins **1**–**4** were purified by HPLC prior to use and experimental details are given in Supporting information. Dose- and time-dependent spermicidal effects of each peptide **1**–**4** were determined in vitro by repeated Sander–Cramer assay.[13] Semen samples were collected from Simmental bulls by penile electroejaculation, from Percheron stallions or Welsh Mountain ponies by artificial vagina, from Large White or Duroc boars by manually assisted ejaculation and from anaesthetized Long Evans rats by surgical extraction as described by Klinefelter et al. using a supplemented Hanks' Balanced Salts solution.[14] All semen sample concentrations were adjusted to yield sperm count ranging from 75 to 122×10^6/mL, motility 70% to 95%, morphology 43% to 92% and viability 50% to 86%. For bovine and horse/pony sperm tests, 800 μg/mL of each bacteriocin was tested as this is the maximum amount that could be solubilized in the testing medium

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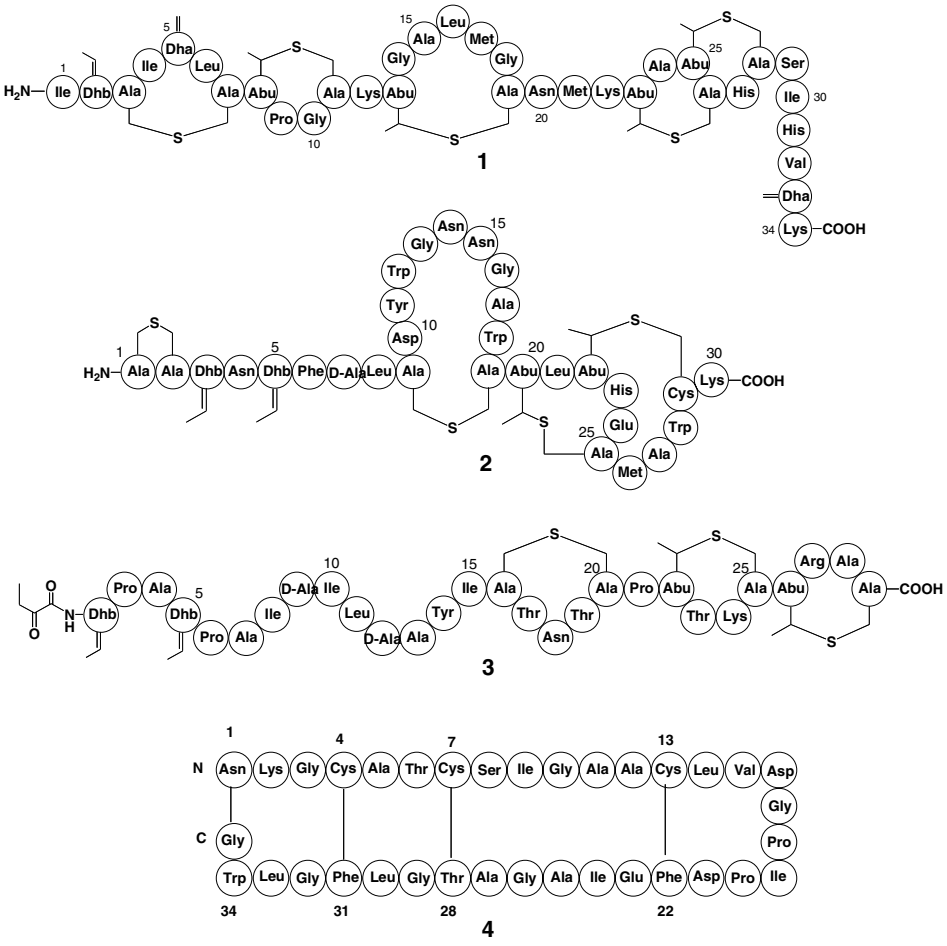

Figure 1. Bacteriocins investigated in this study: nisin A (**1**), LtnA1 (**2**) LtnA2 (**3**) and subtilosin A (**4**).

used (10% DMSO in Ringer's solution). Unlike bovine and horse/pony sperm, boar and rat sperm were found to be completely incompatible with a range of organic solvents, including 10% DMSO. Therefore, 200 μg/mL of each bacteriocin was tested in Ringer's buffer for the boar sperm and in supplemented Hanks' Balanced Salts for the rat sperm. Each sample showing arrest of sperm motility was further analyzed using eosin–nigrosin staining. Only samples that showed sperm immobilization in all of 10 consecutive fields were recorded as a 'pass'. Furthermore, glucose was used in a sperm revival test to ensure that sperm could not regain motility, as described by Aranha et al.[5]

The bacteriocins **1**–**4** were found to exhibit similar spermicidal effects on both bovine and horse/pony spermatozoa (Figs. 2 and 3).

For both species, LtnA1 is the least effective spermicide. Interestingly, LtnA2 is a considerably better spermicide than LtnA1 and shows similar sperm mobility inhibitory effects to nisin A. Subtilosin A was also found to be a moderately potent spermicide against both bovine and horse/pony spermatozoa (Figs. 2 and 3).

In contrast, boar sperm reacted quite differently to the bacteriocins **1**–**4**, with nisin A (**1**) showing the poorest inhibition of sperm motility (Fig. 4). At 200 μg/mL, ni-

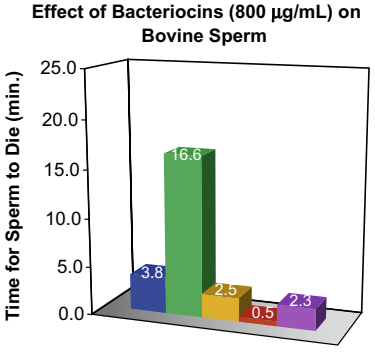

Figure 2. Effect of (800 μg/mL) nisin A (blue), LtnA1 (green), LtnA2 (yellow), LtnA1 + LtnA2 (red) and subtilosin A (purple) on bovine sperm.

sin A (**1**) is virtually ineffective at immobilizing pig sperm. However, LtnA1 (**2**) and LtnA2 (**3**) are moderate boar spermicides when used individually, but when tested together, they exhibit excellent spermicidal activity. Subtilosin A (**4**) yields identical activity as lacticin 3147 (the two-component peptides in combination) and both immobilize boar sperm within 30 s.

In order to compare the activities of lacticin 3147 and subtilosin A with those previously published for nisin

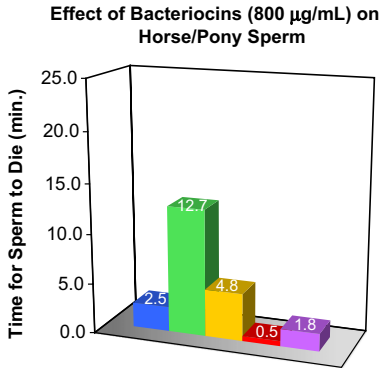

Figure 3. Effect of (800 µg/mL) nisin A (blue), LtnA1 (green), LtnA2 (yellow), LtnA1 + LtnA2 (red) and subtilosin A (purple) on horse/ pony sperm.

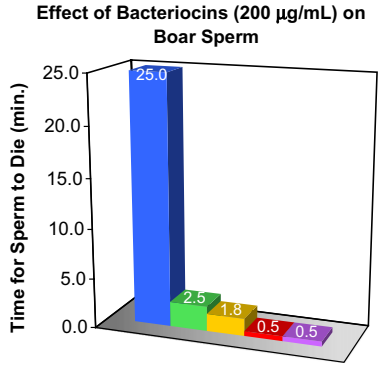

Figure 4. Effect of (200 µg/mL) nisin A (blue), LtnA1 (green), LtnA2 (yellow), LtnA1 + LtnA2 (red) and subtilosin A (purple) on boar sperm.

A, the bacteriocins **1**–**4** were tested against rat sperm. Surprisingly, each bacteriocin has approximately the same effect on rat sperm (Fig. 5). Once again, the lacticin 3147 peptides show the most potent activity, but only when tested in combination. In contrast to published results, in the present work nisin A took 3 min to kill rat sperm at a concentration of 200 µg/mL.

Aranha et al. found that nisin completely immobilized rat sperm in 20 s at a concentration of 50 µg/mL.[5] It is presently unclear as to why the results presented in this study do not match those obtained by Aranha et al., although a few experimental differences, such as testing medium used, type of rats used (Long Evans), method of sperm collection and method of nisin A purification (HPLC purified nisin A was used in this study) may have contributed to the large observed discrepancy. Lacticin 3147 shows the highest spermicidal activity for all animals tested and was analyzed further to determine its minimum inhibitory concentrations (Fig. 6). Interestingly, 200 µg/mL of lacticin 3147 (i.e., 100 µg each of LtnA1 and LtnA2 in combination) instantly kills bovine, horse/pony and rat sperm. As little as 50 µg/mL of lacticin 3147 is able to kill boar sperm.

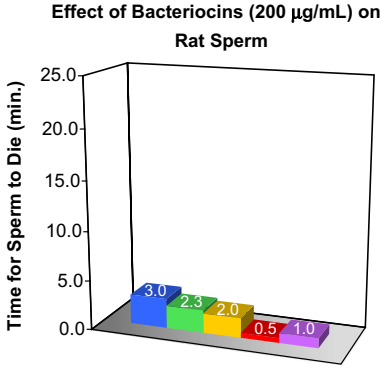

Figure 5. Effect of (200 µg/mL) nisin A (blue), LtnA1 (green), LtnA2 (yellow), LtnA1 + LtnA2 (red) and subtilosin A (purple) on rat sperm.

The exact mechanism by which bacteriocins exert their spermicidal mode of action is currently unknown. Sperm plasma membranes contain high concentrations of anionic phospholipids such as phosphatidylglycerol and phosphatidylserine.[4,15] It is possible that cationic peptides such as nisin A may be attracted to sperm cells via ionic forces and subsequently form pores in their plasma membranes. This would also explain the lack of cytotoxicity of nisin A towards red blood cells, which contain zwitterionic species in their outer membrane, and vaginal cells, which have a low content of negatively charged phospholipids in their outer membrane. In this context it is worth noting that in the study presented here, LtnA2, which is also a positively charged peptide, was found to exhibit a higher spermicidal activity towards bull and horse/pony sperm cells as compared to its neutral partner LtnA1 (Figs. 2 and 3). However, the fact that the neutrally charged bacteriocin subtilosin A was found to be a more potent spermicide than the cationic nisin A (Figs. 2–5) would imply that the spermicidal mode of action of bacteriocins is more complex than ionic interactions. Indeed of it has been proposed by Reddy et al. that nisin A inhibits the activity of the mitochondrial enzyme succinate dehydrogenase (SDH), leading to the inhibition of sperm motility.[6] In a (3-[4-5-dimethylthiazol-2-4]-2,5-diphenyltetrazolium bromide (MTT) assay, MTT is reduced by SDH to give an insoluble, dark blue formazan product. Rabbit sperm cells were first treated with nisin A, then with MTT. Analysis of the resulting solution shows low levels of formazan in the nisin A treated sperm cells, indicating

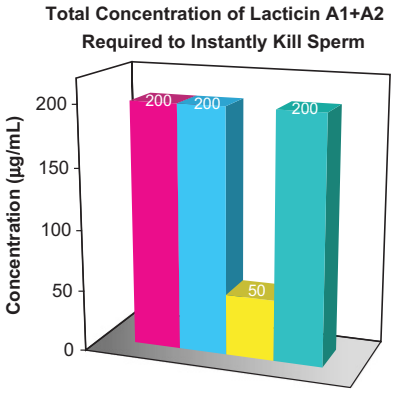

Figure 6. The minimum inhibitory concentration of lacticin 3147 (A1 + A2) required to instantly kill bovine (pink), horse/ pony (blue), boar (yellow) and rat sperm (green).

diminished levels of SDH. It is possible that lacticin 3147 and subtilosin A are also capable of inhibiting this enzyme and this may better explain the observed loss of sperm motility induced by these bacteriocins in this study.

In summary, our results show that the bacteriocins lacticin 3147 and subtilosin A exhibit spermicidal activity against horse/pony, bovine, boar and rat sperm. However, in order for compounds to be useful contraceptives, they must be capable of immobilizing sperm within 30 s. This is because sperm are able to migrate into the cervix and upper reproductive tract within this time frame. In this study, we found that only the bacteriocin lacticin 3147 was capable of immobilizing all the sperm samples analyzed within 30 s. Furthermore, when the two components of lacticin 3147, LtnA1 **2** and LtnA2 **3** were tested individually their spermicidal activity was considerably reduced (Figs. 2–5). This is interesting as this synergistic spermicidal activity observed for LtnA1 and LtnA2 is analogous to the synergistic antibacterial activity exhibited by these AMPs.[8,16] Thus, this result may suggest that the spermicidal activity of lacticin 3147 is due to a specific mode of action rather than a simple surfactant effect.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.11.024.

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