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In vitro spermicidal activity of peptides from amphibian skin: Dermaseptin S4 and derivatives

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Abstract—Sexually transmitted infections and unplanned pregnancies present a great risk to the reproductive health of women. Therefore, female-controlled vaginal products directed toward disease prevention and contraception are needed urgently. In the present study, efforts were made to evaluate the contraceptive potency of dermaseptin DS4, an antimicrobial peptide derived from frog skin. To assess the structure–activity relationship between the native DS4 and its derivatives, a set of chemically modified peptides was synthesized and evaluated. Normal human semen samples were used to detect the spermicidal activity of the new compounds. HeLa cultures were used to determine the safety of compounds toward their toxicity. Fluorescent-binding assays were performed to evaluate the rapidity and the irreversibility of the sperm-immobilizing activity of peptides. All DS4 derivatives elicited concentration-dependent spermicidal activity at microgram concentrations (EC_{100} values: 25 μg/ml–1 mg/ml). The order was K4S4 = S4a > S4 > K4S4(1–16)a > S4(6–28). In cytotoxicity assay, some compounds were found to be significantly safer than nonoxynol-9, the most widely used spermicide, and their activity was not accompanied by total loss of plasma membrane integrity as detected by fluorescent microscopy. Our data also show that increasing the number of positive charges of the peptide resulted in a reduced cytotoxicity without affecting the spermicidal effect. This study indicates that dermaseptins are spermicidal molecules that deserve to be tested as topical contraceptive with useful activities that can add to their prophylaxis, safety, and effectiveness.

1. Introduction

Sexually transmitted infections and contraception are two great concerns in reproductive health of women. Thus, finding products with a double activity, from one hand antimicrobial/antiviral properties with a role in the reduction of sexually transmitted diseases (STD), and from the other hand, spermicidal action to be used as a contraceptive, is an 'ideal' challenge. Moreover, consumer preference studies suggest that most women worldwide prefer using vaginal contraceptive agents that have simultaneously a contraceptive and antimicrobial activity.[1] At the present time, hundreds of spermicidal products have been marketed. Most of them contain a detergent as an active ingredient,[1] such as nonoxynol-9 (N-9), and benzalkonium chlorides. Some of these detergents have also exhibited microbicidal activity in vitro.[2] The spermicidal activities of these surfactants are associated with their structural affinity to the lipid membranes.[3–5] However, numerous laboratory studies have now shown that detergent spermicides do not provide any protection against STD, and their effect in preventing human immunodeficiency virus (HIV) transmission remains controversial.[6] The major drawback of using N-9 or other currently used surfactants is their detergent-like action on epithelial cells and normal vaginal flora.[7–10] The repeated use of a surfactant as a vaginal contraceptive/microbicide has been associated with an increased risk of vaginal or cervical infection and irritation or ulceration.[11–14] Detergent-type spermicides alter vaginal bacterial flora, and such disturbance of the vaginal microbial milieu can lead to opportunistic infections,[15–17] which in turn increases the chance of HIV/STD transmission.[7,18] Due to this effect, it is important to identify and evaluate a new generation of contraceptive antimicrobial agents that act by a different mechanism of action, and can be used vaginally in effective doses without causing overt vaginal irritation or other toxicity.

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Antimicrobial peptides are produced by both prokaryotic and eukaryotic cells and have been the subject of many studies in recent years because of their involvement in host defense mechanisms and their potential use as therapeutic agents.[19–26] These antimicrobial peptides include dermaseptins, a family of eight closely related peptides that were originally isolated from the skin of a tree-dwelling, South American frog (*Phyllomedusa sauvagei*). These compounds are linear polycationic peptides, composed of 28–34 amino acids, which are structured in amphipathic α-helices in apolar solvents.[27,28] They all have a conserved Trp residue at position 3, an AG(A)KAAL(V/G)G(N/K)AV(A) consensus motif in the mid-region, and a positive charge attributable to the presence of Lys residues that punctuate an alternating hydrophobic and hydrophilic sequence.[29] Recently, the identification of new members of the dermaseptin S family (S_9–S_{11}), that do not resemble any of the naturally occurring antimicrobial peptides characterized to date, has been reported after cloning from a skin secretion-derived cDNA library.

Some dermaseptins show a remarkable ability to inhibit microbial cells efficiently, rapidly, and irreversibly without a toxic effect upon mammalian cells. Dermaseptins display cytolytic activity in vitro, generally against a broad spectrum of host-free microorganisms, including bacteria (Gram-positive and Gram-negative),[28–33] protozoa (*Leishmania mexicana* and *Plasmodium falciparum*),[34–37] yeasts and filamentous fungi (*Aspergillus fumigatus* and *Aspergillus niger*)[20,29,38], and viruses such as herpes simplex virus type I (HSV-1)[39] and human immunodeficiency virus (HIV).[40]

The antimicrobial action of dermaseptin is thought to be mediated by interaction of the amphipathic α-helix with the membrane phospholipids, resulting in lethal cell permeation.[20,21,29–32] In this respect, these agents are similar to other families of amphipathic helical peptides such as bombinins and magainins, which are potentially active against a large spectrum of microorganisms.[19,21,41] Although the precise mechanism of action of antimicrobial peptides is not fully understood, they are believed to permeate the target cell by destabilizing the plasma membrane via either a 'barrel-stave' mechanism or a 'non-pore carpet-like' mechanism.[42] Recently a study showed that dermaseptins may have evolved a mechanism to induce cell suicide in invading fungal pathogens. In fact, yeast exposed to dermaseptin S3,[1–16] a truncated derivative of dermaseptin S3 with full activity, showed diagnostic markers of yeast apoptosis: the appearance of reactive oxygen species and fragmentation of nuclear DNA.[43]

Yet, many of these peptides are inactive upon normal eukaryotic cells, but there are some exceptions. It has been previously reported that hamster[44] and human[45] spermatozoa are sensitive to magainins and their motility is reduced.

Structure–activity relationship studies performed on native dermaseptin S4 led recently to the identification of synthetic derivatives with improved antimicrobial properties.[33,46,30] When comparing rates of emergence of resistance by propagating bacteria under selective antibiotic pressure, it was found that both Gram-positive and Gram-negative bacteria exhibit resistance to commercial antibiotics but not to the L- or D-isomers of all dermaseptin derivatives tested.[30] Overall, the data obtained from in vitro and in vivo experiments indicate that some dermaseptin derivatives could be very useful in a variety of antimicrobial applications,[37] especially against opportunistic fungal infections such as *Candida*, which is the most commonly encountered fungal pathogen in the human vagina. These applications are of interest due to the lack of resistance to these agents and their rapid killing mechanism, on the other hand, protection against these infections is highly desirable in topical preparations for vaginal use. Thus, these molecules need to be evaluated as new contraceptive candidates with additional protection against sexually transmitted diseases (STDs).

With the aim to develop a vaginal contraceptive with broad spectrum microbicide activity, we previously evaluated the spermicidal activity of dermaseptin S4 that proved to be high.[47] However, at its active dose dermaseptin was also cytotoxic.[41] In the present work, we developed new dermaseptin-derived peptides with a reduced cytotoxicity and a high spermicidal activity. A structure–activity relationship of cytotoxicity toward a human cervical cell line and sperm plasma membrane for these compounds is presented.

2. Results

2.1. Biochemical modifications enhanced the spermicidal potency of dermaseptin DS4

We used a modified Sander and Cramer's test to evaluate the spermicidal effect of DS4 derivatives.[48] As shown in Figures 1 and 2, exposure for 20 s at 37 °C of highly motile sperm to the peptide resulted in a dose-dependent inhibition of sperm motility with an EC_{100} value of 100 μg/ml. Previously we reported that DS4 was a potent spermicidal compound at 100 μg/ml[47] however, at this concentration, the molecule was significantly cytotoxic.[39] For this reason, we modified DS4 peptide with the aim to reduce its toxicity without affecting its spermicidal activity.

To identify peptides with an improved spermicidal activity without high cytotoxicity, different substitutions and/or deletions were introduced on dermaseptin S4 peptide (Table 1). These included one substitution derivative in which Lys (K) replaced Met in position 4, K4S4, and/or amidation of the peptide, S4a. A second derivative was prepared, wherein the primary structure of dermaseptin S4 was sequentially shortened from the N and/or C termini S4(6–28). A combined substitution and deletion derivative composed of substituted shortened versions of dermaseptin S4 was also prepared K4S4(1–16)a.

These compounds were tested for their spermicidal activity at 10 different concentrations ranging from 5 to 500 μg/ml using the Sander–Cramer test as described

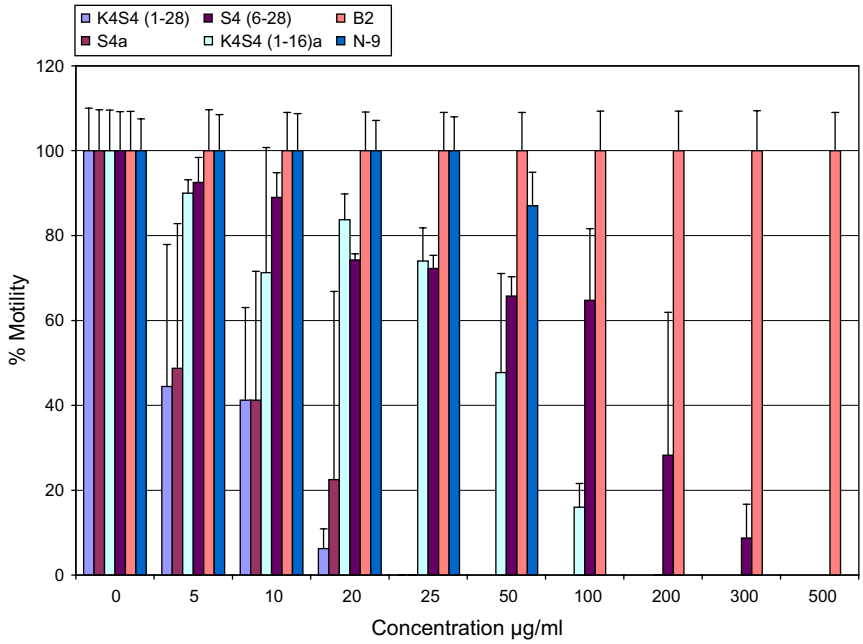

Figure 1. Concentration–response curves showing the effects of five dermaseptins (S4) derivatives on human sperm motility. Highly motile fraction of sperm was incubated for 20 s with increasing concentrations (5–500 µg/ml) of peptides: S4, S4a; K4S4; K4S4(1–16)a and S4(6–28), in presence of negative (medium B2) and positive (N-9) control. The percentages of motile sperm were evaluated by Sander–Cramer modified assay. Each point represents the mean ± SD of three separate experiments.

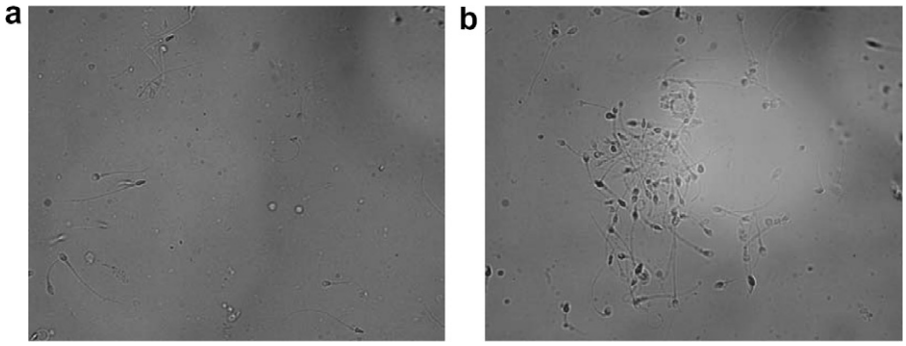

Figure 2. Effect of dermaseptin on sperm motility. Motile spermatozoa were preincubated in the presence or absence of increasing concentrations of five dermaseptins derivatives for 20 s photomicrographics (a) control and (b) in the presence of dermaseptin; complete sperm immobilization was seen after treatment. Original magnification 100×.

Table 1. Sequences of dermaseptin S4 and derivatives

Peptide[a]	Sequence amino acid[b]
S4	ALWMTLLKKVLKAAAKAALNAVLVGANA
S4a	_ _NH2
K4S4(1–28)	_ _ _ K _
K4S4(1–16)a	_ _ _ K _ _ _ _ _ _ _ _ _ _ _ _ NH2
S4(6–28)	_ _

[a] Amide.
[b] A dash indicates that at the specified position, the peptide contains the amino acid identical to that of dermaseptin S4 listed above.

above. As shown in Figure 1, various dermaseptins inhibited sperm motility in a dose-dependent manner. Marked differences were noted in their potency with K4S4 = S4a > S4 > K4S4(1–16)a > S4(6–28), with their EC_{100} in microgram range, at 25 µg/ml for the two more potent peptides.

Among the different dermaseptins, the substituted analog K4S4 was the most active. Inhibition of motility was found to depend on the nature of the peptide, in that the highly charged species were the most active. Thus analog whose positive charge was increased without shortening the length of the peptide was the most potent K4S4, whereas analogs whose positive charge was decreased were less potent such as S4. Likewise, N-terminal deletion S4(6–28) weakened the spermicidal potency and resulted in inability to immobilize all spermatozoa up to the highest concentration assayed (500 µg/ml).

Interestingly, S4a was more active than the native molecule S4, suggesting that C-terminal amidation enforces the spermicidal activity. Deletions of the C-terminal region dramatically affected the spermicidal activity even in presence of substitution and amidation. The spermicidal

activity of dermaseptin was found to be time dependent. Figure 3a and b shows, for the two more potent peptides, that increasing the incubation time increased the spermicidal activity at lower doses. That is illustrated by K4S4, which immobilizes 100% spermatozoa at a dose of 20 μg/ml in only 5 min; whereas almost complete immobilization takes 15 min at a dose of 5 μg/ml. S4(6–28) remains inactive up to 15 min of incubation (data not shown). The kinetics of sperm immobilization thus showed a linear relationship between incubation time and progressive loss of sperm motility after exposure to DS4-derived compounds.

2.2. Dermaseptins affected sperm kinematic

The observed concentration-dependent decrease in sperm motility after exposure to the 4 dermaseptin DS4 derivatives was associated with significant changes in the movement characteristics of the surviving sperm, particularly with respect to VCL; VAP; and VSL. The representative sperm kinematic parameters observed for K4S4 and S4a are shown in Figure 4a and b. However, the linearity (LIN) of the sperm tracks and the straightness (STR) of the swimming pattern were not affected by increasing concentration of the drug; the beat-cross frequency (BCF) and the amplitude of lateral sperm head displacement (ALH) were relatively uniform as the proportion of motile sperm declined with increasing concentration (0–25 μg/ml).

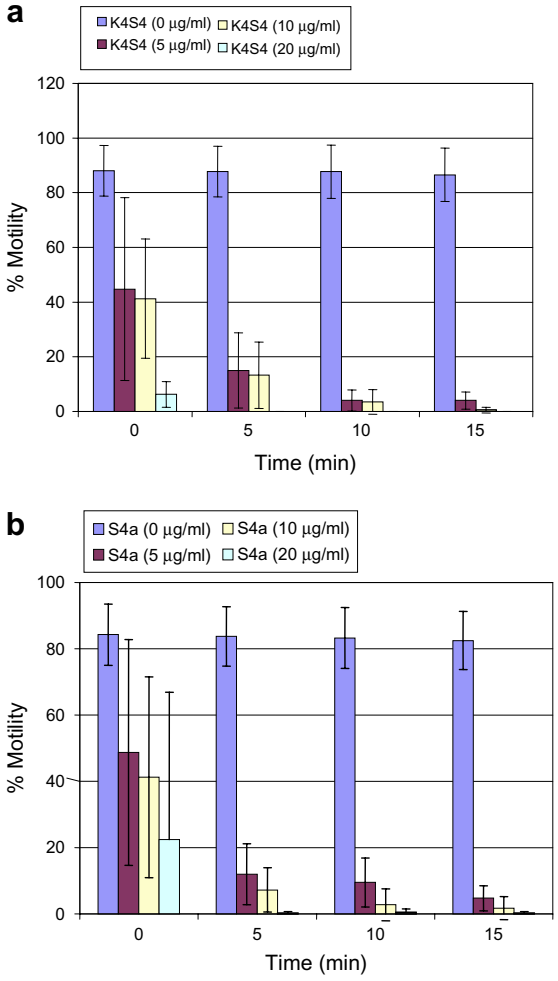

Figure 3. Time-dependent effect of dermaseptin K4S4 (a) and S4a (b) on sperm motility. Motile fractions of sperm were incubated for 0, 5, 10 and 15 min in assay medium in the presence of four increasing concentrations of dermaseptins (0, 5, 10 and 20 μg/ml). Percent of motile spermatozoa was determined by CASA as described in Section 3. Results are expressed as mean ± SD of three-independent experiments.

2.3. In vitro toxicity of dermaseptin and derivatives against human cells

The MTT cell viability assay was used to test the potential in vitro cytotoxicity of DS4 and its derivatives against confluent monolayers of normal human cells (HeLa). Cells were exposed to these peptides at increasing concentrations ranging from 0 to 128 μg/ml. Figure 5 shows that the cytotoxic effect of dermaseptins was concentration dependent. Significant cytotoxicity of all S4 derivatives was observed at concentrations higher than 16 μg/ml, except for S4(6–28) which appeared slightly less toxic than the other peptides.

2.4. Cellular site of interaction of dermaseptins within spermatozoa

Human spermatozoa in suspension treated with FITC-conjugated K4S4 analog were labeled on the flagellum, precisely at the principal piece level whereas the mid-piece and the head were rarely stained (Fig. 6). No difference was observed between 1 and 30 min of incubation time, indicating that the uptake happens in less than 1 min. After washing, the peptide was not removed and cells remained labeled attesting for a strong insertion.

We have previously observed that DS4 was localized both in cell membrane and in the cytoplasm of P4-CCR5 nucleated fibroblastic cells.[40] Because currently used spermicidal compounds are believed to immobilize sperm as a result of detergent-type action on the sperm plasma membrane, we examined the potential sperm membrane damages induced by DS4 at spermicide doses. When human spermatozoa were incubated with fluorescent peptide K4S4 and anti-tubulin antibody, we could observe a preferential loss of the membrane integrity at the flagellum extremity (arrow in Fig. 7) with a less frequent attack of the anterior part of the flagellum. That is illustrated on Figure 7 where on the half anterior part of the flagellum the peptide was present (FITC/green) on an intact flagellar membrane (no red fluorescence indicating an absence of permeability to anti-tubulin antibody) whereas on the flagellum extremity (arrow) the plasma membrane is permeabiliszed/lost, allowing microtubule extrusion.

A double staining of the acrosome (with FITC-PSA) and flagellum (with anti-tubulin) of spermatozoa incubated in the presence of non-fluorescent K4S4 also revealed a loss of the flagellar membrane integrity whereas the matrix and membrane of the acrosome remained intact as attested by the FITC-PSA staining on sperm head (Fig. 8). By contrast, previous studies have demonstrated that sperm exposed to 100 μM of

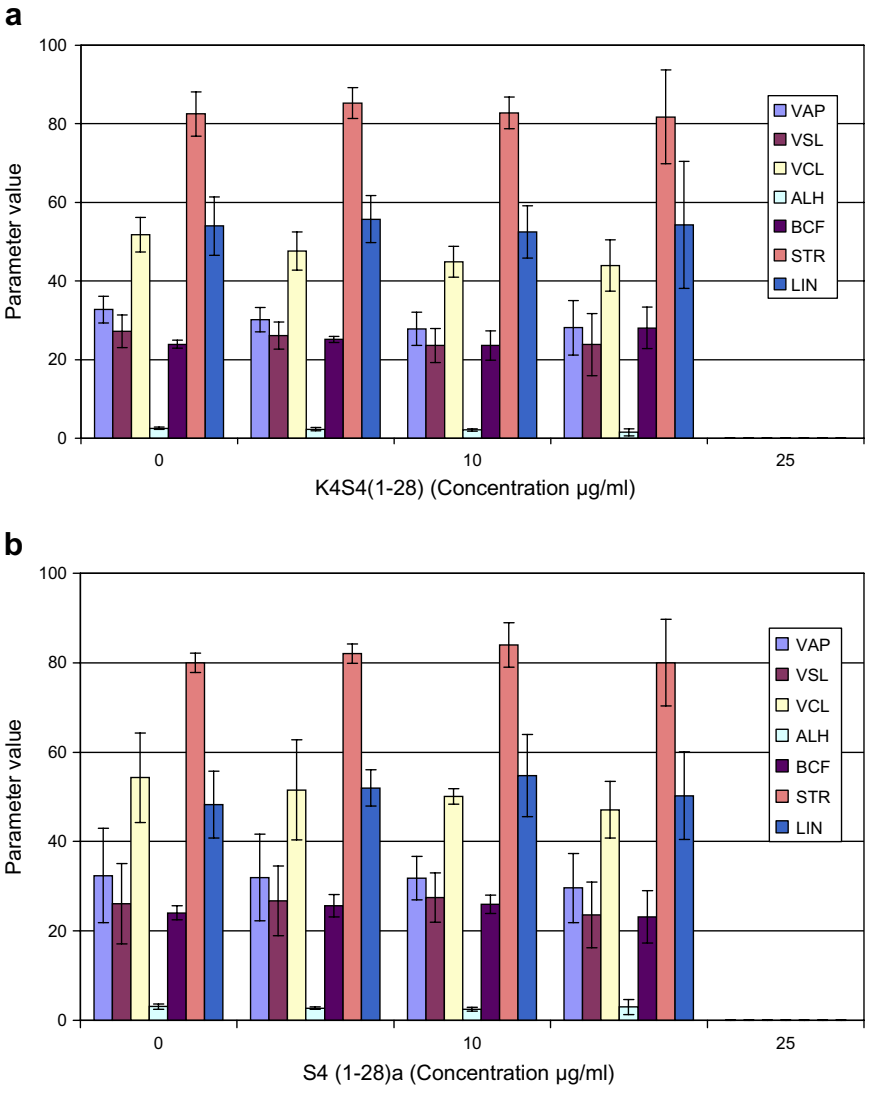

Figure 4. Effect of dermaseptin K4S4 (a) and S4a (b) on sperm motion parameters analysed by CASA. Motile fractions of sperm were incubated in assay medium in the presence of five increasing concentrations of dermaseptins (0, 5, 10, 20 and 25 µg/ml) at 37 °C, and the motility characteristics were determined using the Hamilton–Thorne–Ivos version 10 CASA as described in Section 3. Values for VAP, VSL, and VCL are expressed in µm/s; values for ALH and BCF are expressed in µm and Hz, respectively; values for LIN and STR are expressed in %. Values are means ± SD of three representative experiments. Asterisk denotes a significant difference ($p < 0.05$) between control and S4 treated sperm.

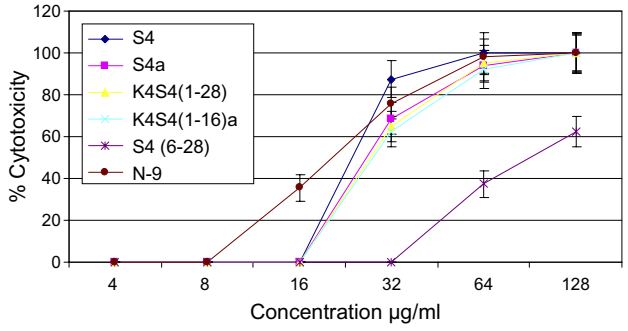

Figure 5. Dose-dependent effect of different derivatives of dermaseptin on viability of normal human cervical cell (HeLa). Cells were incubated with serial dilutions of dermaseptins for 24 h in presence of positive control (N-9), and the conversion of MTT dye to formazan was measured by spectrophotometer. Data represent means of three-independent experiments.

nonoxynol-9 for 3 h under identical conditions revealed complete loss of acrosomal staining with FITC-lectin.[14]

2.5. Discussion

STDs including AIDS pose a serious threat in those heterosexual contacts where the major concern is usually an unwarranted pregnancy. Thus, an effective and consistent use of microbicide during every sexual act requires it to have an in-built contraceptive activity as well. Besides, contraceptive antimicrobial agents are of particular clinical interest because women worldwide prefer a vaginal prophylactic product that is both antimicrobial and contraceptive.[13]

Here, we describe the spermicidal activity of five related synthetic peptides derived from the natural antimicrobial

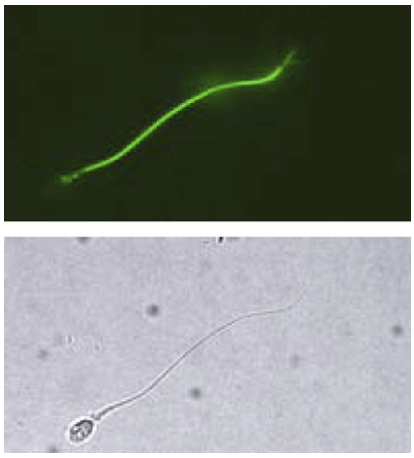

Figure 6. Localisation of interaction site of spermicidal dermaseptin on human spermatozoa. Spermatozoa were incubated with FITC-conjugated K4S4 at spermicidal dose 25 μg/ml. After washing in PBS/BSA, cells were examined using FITC filter of an epifluorescent microscope (top picture) or phase contrast light (bottom picture). Peptide adherence/insertion on spermatozoa was principally observed at the flagellum level.

peptide dermaseptin S4. Our results provide unprecedented evidence that these peptides have potent and irreversible spermicidal activity against human sperm and this effect is dose- and time dependent. Very short exposure to dermaseptins at specific concentrations was sufficient to induce total sperm motility loss, with the most potent dermaseptin described herein immobilizing spermatozoa within 20 s at 25 μg/ml. Prolonged exposure of membrane-intact live sperm can significantly decrease the EC_{100} of peptides. CASA revealed that the observed spermicidal activity of dermaseptin was related to their effects on the VCL, VAP, and VSL of sperm. The spermicidal potency of these peptides may provide the basis for the development of novel vaginal contraceptives.

We observed that dermaseptin K4S4 displayed a four times higher increase in spermicidal potency than the native peptide. It demonstrates that the substitution of methionin by lysine in position 4 without shortening the length of the peptide increased the spermicidal activity of this derivative compared with the native molecule. It was previously shown that increasing the number of positive charges (6 vs 4 for dermaseptin S4) and reducing its hydrophobicity index (HI) (5.5 vs 11.3 for dermaseptin S4) resulted in reduction of hemolytic activity.[20] In addition, we also demonstrated that dermaseptin K4S4 could be a potential anti-HIV microbicide candidate able to disrupt viral particles before infection.[40] The same data also showed that this peptide reduces HIV-1 attachment to human endometrial cells (HEC-1) and HIV-1 transcytosis through a tight HEC-1 monolayer. Moreover, dermaseptin K4S4 presented an improved toxicity profile, although human endometrial epithelial cells are less sensitive to the toxic effect of dermaseptins than other cells.[24] This is an important point because these cells would be the major cell population in contact with a topical microbicide.

Additionally, we observed that neutralization of the negative charge at the C-terminus by conversion of the carboxylate to a carboxamide leads to potent peptide S4a with higher spermicidal activity and slightly lower toxicity against human cervical cells when compared

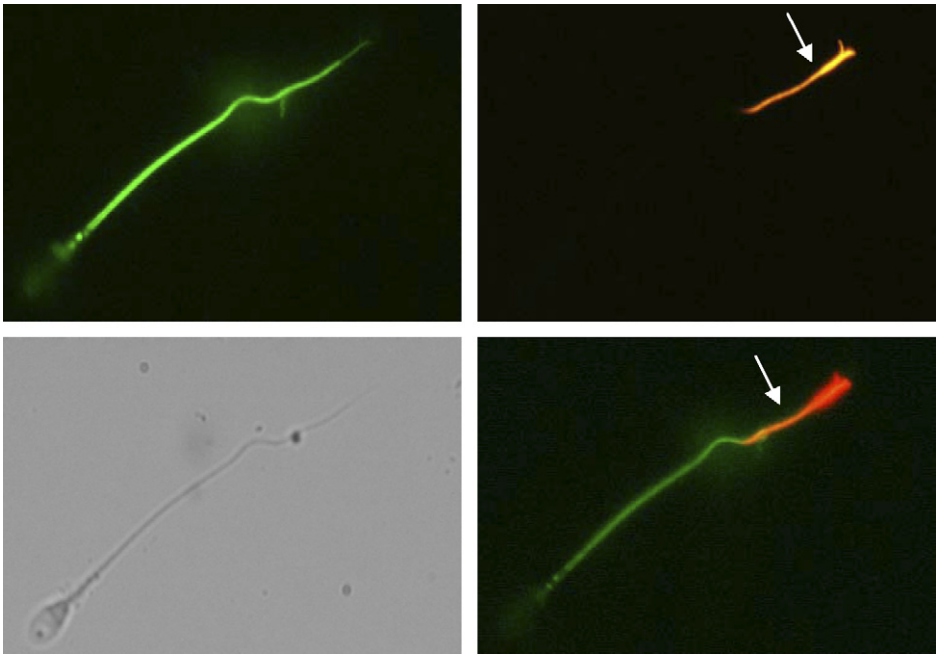

Figure 7. Double labelling of sperm by FITC-conjugated peptide (green) and the mouse anti-tubulin antibody (CY3/red). Spermatozoa were incubated for 10 min at 37 °C with FITC-conjugated peptide K_4S_4 then were washed and stained with anti-tubulin antibody revealed by CY-3-conjugated secondary antibody. Fluorescent K4S4 was observed interacting with sperm flagellum (FITC staining in high left panel). This peptide fixation induced a lost of membrane integrity at the flagellar extremity as revealed by the staining by anti-tubulin (CY-3 fluorescence: arrow in high right panel). In low panels are presented the double FITC/CY-3 staining (right) and the corresponding picture in phase contrast light (left).

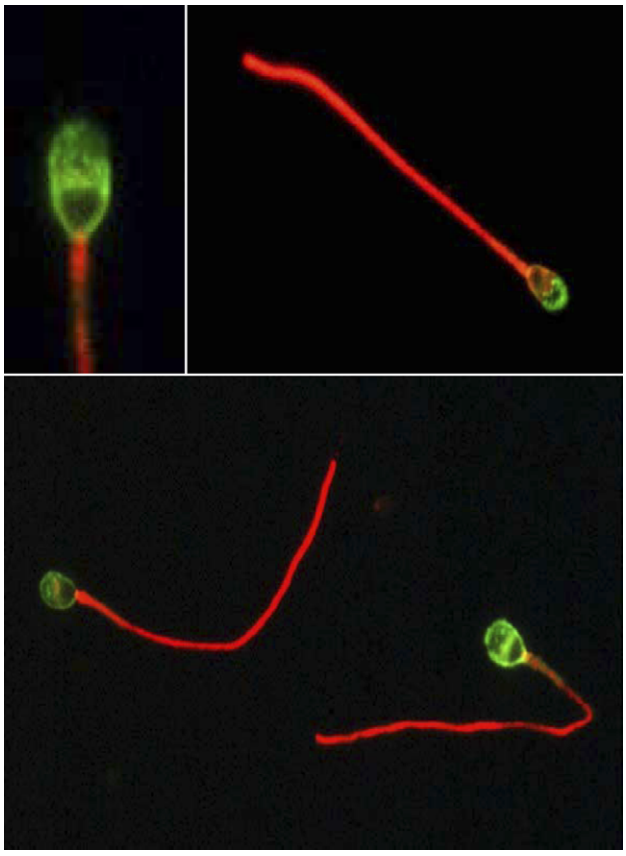

Figure 8. Effect of spermicidal dermaseptins on sperm acrosome and flagellar plasma membrane integrity. Motile sperm were preincubated in the presence of spermicide solution (EC_{100}) of K4S4 and was double labelled with fluorescein isothiocyanate-conjugated *P. sativum* Agglutinin for acrosome labelling (FITC/green) and mouse anti-tubulin antibody for the flagellum staining (CY3/red). The sperm heads exhibited a 'dotted' green fluorescence attesting for the presence of an acrosome. At the flagellar level, we observed a loss of membrane integrity since the anti-tubulin antibodies have reached their axonemal microtubule target.

to S4. Whereas S4a seems to be as potent as K4S4 when spermicidal effect was concerned.

By contrast, the combination of the deletion of the 12 C-terminal residues with the substitution of methionin by lysine in position 4 and the neutralization of the negative charge leads to a peptide, K4S4(1–16)a, with a weak spermicidal activity. However other studies have demonstrated that dermaseptin K4S4(1–16)a had a good anti-HIV activity, as well on HeLa P4-CCR5 cells as on primary PBMCs, had a good antimicrobial activity in vivo with no toxicity in mice, and demonstrated that this analog had reduced cell toxicity at high concentrations.[40] In the present study, dermaseptin K4S4(1–16)a was effective against human sperm but at high concentrations, with a EC_{100} at 200 μg/ml.

Besides, the deletion of 5 N-terminal residues in dermaseptin S4(6–28) had reduced remarkably its spermicidal activity, this peptide is still inactive up to 500 μg/ml. Finally, our study demonstrates that the order of efficacy was K4S4, S4a > S4 > K4S4(1–16)a > S(6–28). Among the five analogs that we tested, dermaseptin K4S4 and S4a showed the most potent activity against human sperm. Based on these observations, we conclude that neutralizing the negative charge of the peptide or increasing its net positive charge results in analogs that display potent spermicidal activity and low cytotoxicity. Our data suggest that the spermicidal effect of dermaseptins is significantly higher than that of other known spermicidal agents including sodium lauryl sulfate[11]; nisin,[49] gramicidin, and magainins.[45] For example, the concentration of these compounds required to immobilize human spermatozoa within 20 s was approximately 2 mg/ml,[45] whereas 100 μg/ml of DS4 (native molecule) was enough to achieve the same effect. However the activity of these compounds against STD pathogens has not been evaluated and no data have shown their ability to inactivate HSV or HIV infections. Comparatively, dermaseptin with the antimicrobial and spermicidal properties may certainly be beneficial as a safe vaginal contraceptive agent for the control of STDs/HIV infections.

Of particular concern is N-9, the most widely used spermicide worldwide. In vitro studies have shown that N-9 had multiple potentially deleterious effects on human endometrium including effect on endometrial structure, perturbations in cytokine production, and damage to the protective endometrial mucin layer.[50,51] These in vitro findings suggest that N-9 can interrupt the functional barrier provided by the endometrium and facilitate infection with HIV and other pathogens. Besides, the spermicidal property of nonoxynol-9 is due to its detergent-like ability to damage the sperm plasma membrane, perturb its conformation, and destroy its semipermeable nature which may lead to a lower degree of protection from STD.[9] Therefore, it may be important that any proposal on improvement in vaginal contraceptives takes into account the non-specific membrane toxicity mediated by detergent-type action of the currently available vaginal contraceptives. Our finding that the acrosome structure of immobilized sperm remains intact demonstrates that the membrane attack by DS4 is restricted and softer than a detergent-like effect.

The mechanism by which dermaseptin exerts its rapid spermicidal action is not known. However DS4, especially K4S4 or Sa, possesses an overall positive charge making them interacting preferentially with anionic phospholipids. As the sperm plasma membrane contains high concentration of phosphatidylglycerol, a strong anionic phospholipid moiety,[51] dermaseptin may have high affinity toward spermatozoa which leads to the loss of permeability of the plasma membrane by forming cation channels that cross cell membrane and induce the cell death. However the exact mechanism by which dermaseptin exerts its spermicidal effect warrants further investigation.

The cytotoxicity assay using the HeLa cell line in vitro indicates that these peptides have their cytotoxicity in almost the same range as N-9.[52] However, some of them such as K4S4 and S4a are significantly more potent (up to four times) than N-9 in their spermicidal property.[52]

Besides, the vaginal epithelial cells which are composed of negatively charged lipids (e.g., cholesterol; phosphatidylserine) are less susceptible than human sperm to dermaseptin.[39] Additionally, the lack of detergent-like membrane toxicity would not increase the susceptibility of vaginal/cervical epithelium to STDs and HIV in repeated use, as seen in the case of N-9.[52] Thus these compounds will be required to be used at a much lower concentration than in vaginal preparations and therefore will prove to be comparatively much safer.

Finally, dermaseptin DS4 derivatives present a new lead structure for potent spermicidal agents with complementary properties that add to their utility as safe, effective, and prophylactic topical contraceptives.

Multiple biochemical modifications on the native peptide structure yield molecules with ideal bioactivity and low toxicity that can replace surfactant-type spermicides such as N-9 in vaginal contraceptives, making them more safe and acceptable. Therefore, DS4 derivatives deserve to be further evaluated in animal models to test their toxicity in mucosal tissues and their ability to be a barrier contraceptive agent both alone or in combination with other potential compounds. Studies are underway in this direction.

3. Materials and methods

3.1. Peptide synthesis

Peptides were prepared by stepwise solid phase synthesis using F_{moc} polyamide-active ester chemistry on Milligen 9050 pepsynthesizer. All F_{moc}-amino acids were from Milligen-Waters (France). 4-(Hydroxymethyl) phenoacetic acid-linked polyamide/kieselguhr resin (pepsin kA), Fmoc-aminoacid pentafluorophenyl (Pfp), and 3-hydroxy-2,3-dehydro-4-oxo-benzotriazine (Dhbt) esters were from Milligen/Bioresearch. Cleavage of peptidyl-resin and side chain deprotection were carried out using 5 mg of peptidyl-resin in 1 ml mixture composed of trifluoroacetic acid, *para*-cresol, thioanisol, water, and ethyl methyl sulfide (82.5; 5.5; and 2.5% v/v) for 2 h at room temperature. After filtering to remove the resin and ether extraction, the crude peptides were purified by a combination of Sephadex gel filtration, ion exchange chromatography, and preparative high performance liquid chromatography (HPLC). Homogeneity of synthetic peptides was assessed by analytical HPLC, amino acid analysis, solid phase sequence analysis, and mass spectrometry.[27] All peptides were stored frozen as stock solutions at 1 mg/ml in double-distilled water at −20 °C. Prior to experimentation, fresh solutions were diluted in the appropriate medium. For fluorescein-labeled peptides, fluorescein was introduced at the N-terminus of the peptide using fluorescein *N*-hydroxysuccinimide ester prior to TFA treatment.

3.2. Motile human sperm preparation

Fifty fresh human semen samples collected by masturbation were obtained from patients ranging in age from 23 to 35 years undergoing routine semen analysis at the laboratory of Biology of the Reproduction and Cytogenetics in Cochin's Hospital, Paris. The samples were allowed to liquefy at 37 °C for 30 min. The semen characteristics, volume, pH, viscosity, and sperm morphology, were determined according to World Health Organization guidelines.[53] The remainder of the sperm was diluted 1:1 with B2 (INRA Medium, France) layered on discontinuous (90–45%) Percoll gradient and centrifuged at 300*g* for 20 min at room temperature. The pellet of selected motile spermatozoa was washed by suspension in 5 ml of Earle medium (Sigma–Aldrich) supplemented with 1% (w/v) BSA (Sigma–Aldrich) and centrifugation (600*g* for 10 min) and was resuspended in 1 ml aliquots in B2.

3.3. Sperm immobilization assay

To evaluate the spermicidal effect of derivatives of dermaseptins (DS), S4; K4S4; K4S4(1–16)a; S4a S4(6–28), a highly motile fraction of sperm (20×10^6) prepared from pooled donor sperm was incubated in 1 ml of B2 containing serial dilutions of the test compound.

The spermicidal action was determined on spermatozoa separated from seminal plasma by studying the effect of various concentrations of DS derivatives (5, 10, 20, 25, 50, 100, 200, 300, and 500 μg/ml) on sperm motility evaluated by the Sander and Cramer test.[48] Briefly, 1 ml of spermicidal compound was mixed with 0.2 ml of motile sperm fraction at 37 °C. Rapidly after mixing (20 s), a sperm aliquot was taken and microscopically analyzed for percentage and grade of sperm motility. The weakest concentration (or highest dilution) that completely immobilized all the spermatozoa within 20 s was recorded as EC_{100}. This latter parameter expressed the spermicidal activity of DS4 and its derivatives.

To test the effect of incubation time on spermicidal activity of dermaseptin DS4 and its 4 derivatives, a motile fraction of sperm (20×10^6/mL) was incubated at 37 °C in B2 in the presence of different concentrations of DS4 derivatives ranging from 0 to 500 μg/ml for S4(6–28); 0 to 20 μg/ml for K4S4 and S4a; and 0 to 100 μg/ml for S4 and K4S4(1–16)a.

At time intervals of 5 min, duplicate aliquots (5 μl) were transferred to two Microcell chambers and sperm motility was assessed by CASA (Computer Assisted Sperm Analysis, IVOS version 10 instruments Hamilton Thorne Research, Danvers, MA) for duration of 15 min.

3.4. Sperm kinematic parameter measurements

Aliquots (0.1 ml) of human semen containing highly motile fraction of sperm ($>10 \times 10^6$/ml) were suspended in twofold diluted B2 medium and semen samples were analyzed by CASA for inhibition of sperm motility. For CASA, five microliters of each sperm suspension ($>10 \times 10^6$/ml) was loaded into two 20 μm-depth Microcell chambers maintained at 37 °C with a stage Warner. At least 8–10 fields per chamber were scanned under phase-contrast illumination for analysis using a Hamilton Thorne integrated visual optic system. Each field

was recorded for 1/2 s at a frame rate of 60 frame/s. Other settings were as follows: minimum contrast: 8; minimum size: 6; low-size gate: 0.6; high-intensity gate: 1.4. The performance of the analyzer was periodically checked using the playback function.

The kinematic parameters determined for each sperm sample included number of motile (MOT) and progressively (PRG) motile sperm; curvilinear velocity (VCL; total distance travelled by sperm head divided by the recording time, in μm/s); average path velocity (VAP; the spatially averaged path of VCL that eliminates the wobble of the sperm head in μm/s); straight-line velocity (VSL; the straight-line distance from beginning to end of track divided by time taken, in μm/s); beat-cross frequency (BCF; frequency at which sperm head crosses sperm average path); amplitude of lateral head displacement (ALH; the mean width of sperm head oscillation, in μm); and the derivatives straightness (STR = 100 × VSL/VAP) and linearity (LIN = 100 × VSL/VCL). At least 200 motile spermatozoa were analyzed for each sperm sample.

3.5. Assay for cell viability

The potential cytotoxicity of dermaseptin S4 and its derivatives against normal human cervical cell (HeLa) was measured by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide)-based assay. Briefly, cells were seeded into 96-well plates at a density of 2×10^4 cells per well and incubated for 24 h at 37 °C prior to drug exposure. On the day of treatment, culture medium was aspirated from the wells and replaced with the fresh medium containing drug concentration ranging from 0 to 500 μg/ml; triplicate wells were used for each treatment. Culture plates were then incubated for 30 min, 3 h or 6 h before 10 μl of MTT solution (5 μg/ml in PBS) was added to each well. The wells containing only medium and MTT were used as controls for each plate. The tetrazolium/formazan reaction was allowed to proceed for 4 h at 37 °C, and then 100 μl of the solubilization buffer (10% sodium dodecyl sulfate in 0.1% (v/v) HCL) was added to all wells and mixed thoroughly to dissolve the dark-blue formazan crystals. After an overnight incubation at 37 °C, the optical density at 540 nm was measured with 96-well multiscanner autoreader, with the solubilization buffer serving as blank. To translate the OD_{540} values into the number of live cells in each well, the OD_{540} values were compared with those of standard OD_{540}-versus-cell number curves generated for each cell line. The percentage of cell survival was expressed as live cell number in test group/live cell number in control group × 100.

3.6. Localization and membrane effect of the dermaseptin derivative on human spermatozoa

0.1 ml aliquot of liquefied selected motile sperm fraction was treated with an appropriate volume of the fluorescent peptide FITC-conjugated K4S4 at 25 μg/ml (or buffer in control tubes). After 1–30 min of incubation time, cells were washed with PBS/5% (w/v) BSA and examined using an epifluorescent microscope (Nikon). In order to better know the effect on sperm membrane (at sperm membrane level) of these new non-detergent spermicides, we conducted staining assay using two fluorescent markers targeting the acrosome (fluorescein isothiocyanate-conjugated-*Pisum sativum* Agglutinin (FITC-PSA)) and the flagellum (monoclonal mouse anti-tubulin antibody (Sigma)). A positive labeling for PSA, which precisely targets the outer acrosomal membrane and matrix, will indicate a limited plasma membrane damage with the preservation of an intact acrosome in spermatozoa and a positive staining of tubulin will indicate that flagellar plasma membrane integrity is lost, making it permeable to anti-tubulin antibodies. A 0.1 ml aliquot of liquefied motile sperm fraction was treated with 0.1 ml of spermicide solution at the EC_{100} (or 0.1 ml of buffer in control tubes) and incubated for 1 min to 30 min at 37 °C. The spermatozoa were pelleted by centrifugation (600*g* for 5 min) and 0.25 ml of anti-tubulin at a dilution of 1:5000 in phosphate-buffered saline/5% bovine serum albumin (PBS/ BSA)] was added to the pellet and mixed gently. The mixture was incubated for 1 h at 37 °C. After washing, 10 μg/ml FITC-PSA diluted in PBS/BSA was added to the pellet and incubated for 15 min. After a final washing and re-suspension of the pellet in PBS; a drop was observed under ×100 objective, using an epifluorescent microscope (Eclipse 600 NIKON). PSA-FITC and CY3 anti-tubulin were simultaneously detected using the FITC/Texas red double filter.

3.7. Statistical analysis

Data were expressed as means ± SD. For statistical analysis of data, statistica version 5.0 followed by multiple comparison two-tail *t*-test was employed. Differences were considered statistically significant if $p < 0.05$.

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