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Dermaseptins as potential antirabies compounds

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Over the last 20 years, natural peptides playing a key role in defense mechanisms and innate immunity have been isolated from unicellular organisms. Amphibian skin secretes dermaseptins, 24–34 amino acids in length that have a wide antimicrobial spectrum incorporating yeast, fungi, protozoa, bacteria and enveloped viruses. The anti-rabies virus (RABV) activity of dermaseptins S3 (30aa) and S4 (28aa) from *Phyllomedusa sauvagei* has been investigated, and further dissected its molecular basis by comparing punctual mutation or deletion of S4 analogues. The results showed that: (1) S4 is more active than S3 against RABV infection, 89% versus 38% inhibition at 7.5 μ M; (2) the 5 NH₂-aa of S4 are crucial for its inhibitory potential (S4_{6–28} lost any inhibition) but the COOH terminus stabilizes the inhibitory potential (S4_{1–16} showed only 23% inhibition at 7.5 μ M); (3) there is a correlation between viral inhibition and dermaseptin cytotoxicity, which remains however moderated for BSR cells ($\leq 12\%$ at 10 μ M). A single mutation in position 4 (S4_{M4K}) slightly reduced cytotoxicity while keeping its antiviral activity, 97% at 7.5 μ M. S4 and S4_{M4K} showed an antiviral activity *in vitro* when provided 1 h after infection. *In vivo* experiments in mice by intramuscular injection of non-toxic doses of dermaseptin S4_{M4K} 1 h post-infection by a lethal dose of RABV at the same site allowed more than 50% improvement in mice survival. This study highlights the potential interest of dermaseptins as non-expansive alternatives to rabies immunoglobulins for the treatment of rabies that continues to claim about 60,000 human lives per year worldwide, almost exclusively in developing countries.

1. Introduction

Rabies encephalitis remains a serious public health problem worldwide, particularly in developing countries from Asia and Africa. The most recent estimates indicate that the number of human cases is approximately 59,000 per year, mostly due to dog bites, which corresponds to 3.7 million disability-adjusted life years (DALYs) and 8.6 billion USD economic losses per year [1]. This alarming death toll is primarily due to either insufficient availability and/or access to the optimal post-exposure prophylaxis (PEP) recommended by the World Health Organization (WHO) [2]. This prophylaxis comprises a series of 4–5 injections of modern vaccines prepared on cell/tissue culture, most commonly through local instillation of anti-rabies immunoglobulins (RIGs) from

human (HRIGs) or Equine (ERIGs) origin, either complete or in the form of purified F(ab')₂ fragments (F(ab')₂-ERIGs) [1]. It confers protection by combining the induction of an active immune response and the immediate neutralization of the viral inoculate by exogenous neutralizing antibodies. However, many of the biological reagents required, particularly HRIGs and ERIGs, are both scarce and costly for developing countries. Consequently, among the 10 million exposed people receiving the PEP each year, many do not complete the appropriate schedule. Alternative approaches including reduced vaccination regimens, optimized use of antigens, monoclonal antibody cocktails to replace RIGs as well as use of antivirals are being assessed [3–9]. Antivirals are particularly attractive since rabies has a long incubation period (two months in average), during which period no obvious clinical symptoms are observed. However, RABV is neurotropic and not accessible to drugs once within the neurons. Therefore, drugs targeting the early stages of infection, particularly at or close to virus entry into

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epithelial cells, or during the initial replication steps around the biting area, appear promising.

Amphibian skin glands have proven to be a rich source of antimicrobial peptides, with approximately 500 described [10,11]. These peptides include dermaseptins, a large family produced by the skin of tree frogs belonging to the genera *Phyllomedusa* [12–16]. Dermaseptins are linear polycationic peptides, 24–34 amino acids in length, adopting an amphipathic α -helical structure in apolar solvents [17] as well as in membrane environment. This structure is considered to be the pre-requisite for lipid membrane destabilization and lytic activity [18–22]. During the last ten years, dermaseptins have pioneered the conception of novel antibiotics with a large spectrum of action. They exert a rapid cytolytic activity *in vitro* against a broad spectrum of pathogenic microorganisms including bacteria [23–25] protozoa [26–28], yeast, parasites and filamentous fungi [29–31]. With regard to viruses, dermaseptins display an antiviral effect against amphibian viruses including channel catfish virus or frog virus 3 (the causal agent of red leg disease) [32], but also against human viruses such as herpes simplex virus type I and II (HSV-1 and 2) [33,34], human immunodeficiency virus type I (HIV-1) [35,36] and human papillomavirus [28].

Although dermaseptins are principally known for their ability to interact with and destabilize lipid membranes [19,37,38], they can also act internally after membrane crossing. One of the most relevant examples is the dermaseptin S3, which has been shown to interfere with the DNA integrity of *Saccharomyces cerevisiae* [29], and can also affect *Plasmodium falciparum* at the intra-erythrocyte level [39]. For enveloped viruses, however, the viral envelope is the preferential target. The dermaseptin S4 and its derivatives have been shown to exert an anti-HIV-1 and anti HSV-1 and 2 activity prior to viral entry, either by disruption of the envelope of the viral particle or by interacting with viral or cell surface molecules responsible for attachment, adsorption and/or fusion [33–35]. As RABV is an enveloped virus, which penetrates the cell via a receptor-mediated recognition [40–42], endocytosis and membrane fusion, it may also represent a potential target for dermaseptins. In this study the anti-rabies activity of dermaseptins S3, (30aa), S4 (28aa) and S4 derivatives both *in vitro* and *in vivo* has been assessed.

2. Materials and methods

2.1. Cells and viruses

BSR cells, a clone of the Baby Hamster Kidney cells (BHK-21) [43] were cultivated in Dulbecco's modified Eagle's medium (DMEM), Glutamax I (Gibco) supplemented with 5% heat inactivated foetal calf serum (FCS: Sigma) and 40 μ g/ml gentamycin (Sigma). For *in vitro* infection experiments, BSR cells were seeded in 96 well plate (3.5×10^4 cells per well), grown for 24 h at 37 °C under 5% CO₂ until 70–80% confluence was achieved, then infected with the RABV Pasteur virus (PV) strain (master suspension 10^8 UFF/ml) at a Multiplicity of Infection of 1 (MOI = 1). For *in vivo* infection experiments, the RABV "Challenge Virus Strain" (CVS) strain (master suspension 2.25×10^8 UFF/ml) adapted to mouse brain, was used for infecting mice 1 h before inoculation with the dermaseptin.

2.2. Dermaseptins

Dermaseptins used in this study are analogues to those secreted by the skin of *Phyllomedusa sauvagei*, a south-American tree frog. Two dermaseptins (S3, S4) and three S4-derivatives: S4_{M4K} (Met to Lys substitution in position 4); S4_{1–16} (deletion of the 12 COOH

amino-acids) and S4_{6–28} (deletion of the 5 NH₂ amino-acids) were compared (Table 1). The five dermaseptins were chemically synthesized as described in [35], lyophilized and stored at –20 °C. Prior to experiments, fresh master solutions were prepared in sterile water, then diluted in Dulbecco's Modified Eagle Medium (DMEM) with Glutamax supplement (Gibco) at the required concentration before addition to the cell supernatant or in phosphate buffered saline (PBS) medium (Gibco) before injection to mice. Concentrations ranging from 1 to 10 μ M were tested *in vitro* experiments. For *in vivo* experiments in mice, 100 μ g or 200 μ g of dermaseptin (purchased from PEPTIDE2.0, USA) were inoculated in the gastrocnemius muscle.

2.3. Cytotoxicity experiments

A plate of 96 wells was seeded with BSR cells (4×10^4 cells per well) in DMEM-Glutamax medium supplemented with 5% of heat inactivated foetal calf serum (Gibco) and 40 μ g/ml gentamycin. After 24 h at 37 °C under 5% CO₂, 50 μ L of DMEM-Glutamax containing dermaseptin was added to the cell culture supernatant (final dermaseptin concentration ranging from 1 to 10 μ M). After 2 h at 37 °C under 5% CO₂, the amount of lactate dehydrogenase (LDH) released in the supernatant was measured using the Cytotox 96® non-Radioactive Cytotoxicity Assay (Promega). The cytotoxic effect of dermaseptins was quantified as followed: [(LDH in supernatant of treated cells) – (LDH in supernatant of untreated cells)] / (LDH in lysed untreated cells).

2.4. Antiviral activity of dermaseptins

2.4.1. *In vitro*

The antiviral activity of dermaseptins was evaluated pre-, during or post-infection. For all experiments, cells were first cultivated for 24 h at 37 °C in DMEM-Glutamax medium (Gibco) supplemented with 5% of heat inactivated foetal calf serum and 40 μ g/ml gentamycin.

For pre-treatment experiments, the cell supernatant was removed, replaced with fresh medium supplemented with dermaseptin at the desired concentration, and incubated for 1 h at 37 °C under 5% CO₂. The supernatant was then discarded and cells were infected with the RABV PV strain (MOI = 1) in fresh medium. For co-treatment experiments, cells were infected (MOI = 1) and incubated at 37 °C in presence of the desired concentration of dermaseptin. For post-infection treatment, cells were infected (MOI = 1) and incubated for 1 h at 37 °C. Then the virus inoculum was removed and replaced with fresh medium supplemented with the desired concentration of dermaseptin.

All dermaseptins, i.e. S3, S4, S4_{M4K}, S4_{1–16} and S4_{6–28} were evaluated in co-treatment. The two dermaseptins giving the most promising antiviral effect (S4 and S4_{M4K}) were additionally evaluated in pre- and post- treatment.

Table 1

Amino acid sequence of the S3 and S4 dermaseptins and their derivatives. Gaps introduced to improve the similarity in S3 and S4 are indicated by dots. Conserved residues between S3, S4 and S4 derivatives are underlined. Dashes indicate deletions in S4 derivatives. Mutated residue is indicated in bold character. IC₅₀ is the dermaseptin concentration (μ M) that induces 50% infection inhibition of BSR cells.

Dermaseptin	Amino acid sequence	IC ₅₀
S3	<u>ALWKNMLKGGIGKLAGKAALGAVKKLVGABS</u>	8.5
S4	<u>ALWMTLLKKVLEKAAAKAALNAV</u> .LVGANA	5.1
S4M4K	<u>ALWKTLLKKVLEKAAAKAALNAV</u> .LVGANA	5.1
S4 _{1–16}	<u>ALWMTLLKKVLEKAAK</u> -----	>10
S4 _{6–28}	---- <u>LLKKVLEKAAAKAALNAV</u> .LVGANA	>>10

The anti-RABV effect of pre- co- and post-treatment with dermaseptins was evaluated after 24 h of infection at 37 °C in comparison to non-treated cells. The experiments were repeated in duplicate. The modified Rapid Fluorescent Focus Inhibition Test (RFFIT) was used to quantify the infection [44]. Briefly, BSR cells were washed with PBS (Gibco) and fixed with 80% acetone for 30 min on ice. Acetone was removed and the plate dried. Cell infection was revealed using a rabbit polyclonal antibody directed against the RABV ribonucleoprotein conjugated to fluorescein (FUJIREBIO Diagnostics Inc.). The antiviral effect of dermaseptins was evaluated by comparing the percentage of infected cells in mock-treated and treated cells under the same conditions.

Images of infected cells were acquired on an automated spinning disk confocal microscope (Opera QEHS, Perkin Elmer Technologies). Briefly, three fields were acquired per well (above 2000 cells) using a 10× air objective (NA 0.4) and the following settings: (Ex/Em: 405 nm/450 nm) to reveal the cell nuclei stained with DAPI and (Ex/Em 488 nm/540 nm) to detect ribonucleoprotein (RNP). The image analysis was performed using Acapella™ (Perkin Elmer Technologies) using custom developed software. Firstly, the nuclei (DAPI channel) enabled an estimation of the number of cells per well (TN for total nuclei); then a “cell mask” was generated around each nucleus to delimitate each individual cytoplasm; when viral RNP (at least one “green spot”) was ‘spotted’ inside the defined cytoplasm area, the cell was considered “infected” (InfC); the ratio $[\text{InfC}/\text{TN} \times 100]$ corresponded to the percentage of infection per well.

2.4.2. *In vivo*

Animal experiments were performed in compliance with the French animal protection law and in respect of the procedures used at the Institut Pasteur animal facility. SWISS female mice at 6–8 weeks and 25–30 g each (Janvier, France), were divided in experimental groups of eight to ten mice. They were housed with access to food and water *ad libitum* throughout the experiment. Before virus inoculation, the mice were anesthetized by intra-peritoneal injection of 100 µL of a combination of 25% Imalgene 1000 and 12.5% Rompun (Centravet®), diluted in final volume of 1× PBS (Gibco®). Mice were inoculated with virus or dermaseptin in the gastrocnemius muscle. Control groups received different doses of the RABV CVS strain or 100 µg or 200 µg of dermaseptins S4 or S4MAK alone, diluted in DMEM medium. The other groups were first inoculated with the RABV, and 1 h later, with 100 µg or 200 µg of S4 or S4MAK at the same muscle area. The master RABV solution titre was 2.25×10^8 UFF/ml (Unit Forming Unit in BSR cells). Virus suspension was thawed just before mouse inoculation and diluted in DMEM (Gibco) for adjustment at the desired concentration. Mice were monitored daily over 16 days for appearance and development of clinical signs of increased severity, using the following clinical scoring system: (1) ruffled fur, hunched back; (2) slower movements; (3) twitching in inoculated limb; (4) paralysis. At grade 3–4 (day 6–9 post-inoculation) they were terminated according to ethical humane endpoints by CO₂ inhalation.

3. Results

3.1. *Dermaseptins are moderately cytotoxic*

We first evaluated the cytotoxicity in BSR cells of dermaseptins S3, S4 and its three derivatives: S4_{M4K} with a Met to Lys substitution in position 4; S4_{1–16} with a deletion of 12 amino-acids at the COOH extremity; S4_{6–28} with a deletion of five amino acids at the NH2 extremity (Table 1). The cytotoxicity was assessed by quantifying the amount of lactate dehydrogenase (LDH), a cytoplasmic enzyme released in the medium upon cell lysis, using

the Cytotox96® Non-Radioactive Cytotoxicity Assay (Promega). The level of cytotoxicity was calculated as a percentage of the amount of LDH released after complete lysis of BSR cells. Due to the lack of the LDH stability in the extracellular medium, the cytotoxicity of the dermaseptins was evaluated after 2 h of incubation with the BSR cells. In the range of concentrations tested (1–10 µM) the percentage of cell lysis remained moderate, inferior in all cases to 12% of the cells (Fig. 1A). However, it was possible to classify the dermaseptins in three groups. S4 and S4_{M4K} exhibited the higher cytotoxic effect, ranging from 4–6% at 3 µM to 11–12% at 10 µM. A second group comprising S3 and S4_{1–16} demonstrated a lower cytotoxic effect, ranging from 1–2% at 3 µM to 9/10% at 10 µM. Finally S4_{6–28} did not show any cytotoxicity. In summary, the cytotoxicity of S3 and S4 was very moderate and most of the cytotoxicity of S4 is likely due to its five NH2 amino acids.

3.2. *The first five NH2-amino acids of dermaseptin S4 are essential to the anti-RABV activity*

BSR cells were infected with the PV strain of RABV (MOI = 1) in the presence of increasing concentrations of dermaseptins to evaluate their anti-rabies activity. Twenty-four hours post-infection, the percent of inhibition of RABV infection was measured using the RFFIT method. The percent of BSR cell infection in the presence of dermaseptins was calculated by comparison to mock-treated infected BSR cells (considered 100% infection). Fig. 1B illustrates the progressive decrease of infected BSR cells (spots of fluorescent RNP) with increasing concentrations of dermaseptin S4. In terms of antiviral activity, the dermaseptins were classified into three groups based on their anti-RABV activity (Fig. 1C). Both S4 and S4_{M4K} showed the strongest antiviral activity ranking over 85% from 7.5 µM to 10 µM and decreasing progressively to 47% at 5 µM and 14–20% at 3 µM. Dermaseptin S3 and S4_{1–16} were less efficient, inhibiting only 63% and 34% of RABV infection at 10 µM, respectively, while this activity decreased considerably to only 15–11% at 5 µM. Finally, dermaseptin S4_{6–28} did not show any antiviral activity, even at a high concentration. Again, it appeared that the deletion of the first five NH2 amino acids was deleterious to the anti-RABV activity of the dermaseptin S4, supporting the hypothesis that these residues likely concentrate most of the inhibitory potential, in addition to the cytotoxic effect.

3.3. *The dermaseptins remain active in post-infection protocols*

To investigate the therapeutic potential *in vitro* of the two dermaseptins demonstrating the more promising antiviral effect (S4 and S4_{M4K}), we further assessed their ability to inhibit RABV when delivered pre- or post-infection. When dermaseptins remained in contact with BSR cells during the 1 h incubation before removal of the supernatant prior to infection, no significant antiviral effect was observed: only a residual inhibition (<10%) was observed at low concentration (0.5–1 µM) for S4 (Fig. 2A) and 25% of inhibition at high concentration for S4_{M4K} (4 µM) (Fig. 2B). However, when the dermaseptins were added 1 h post infection the antiviral effect was improved, and increased with concentration up to approximately 40% of inhibition with S4 at 4 µM, while S4_{M4K} remained limited to 25% inhibition from 3 to 4 µM. This post-exposure inhibitory effect is significant and comparable to that obtained when the dermaseptins were present during infection (Fig. 1C).

3.4. *In vivo protective activity of dermaseptin*

For *in vivo* experiments, 7 groups of 8 mice each were followed during 16 days. Two groups were inoculated in the gastrocnemius muscle with 100 or 200 µg of dermaseptin S4 or dermaseptin S4_{M4K}. The mice continued feeding normally and did not exhibit

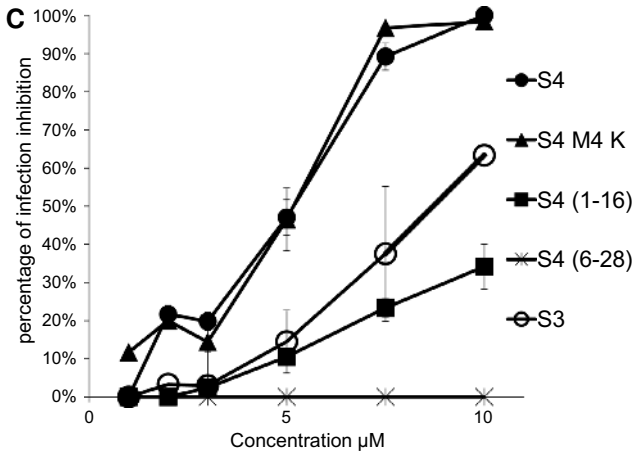
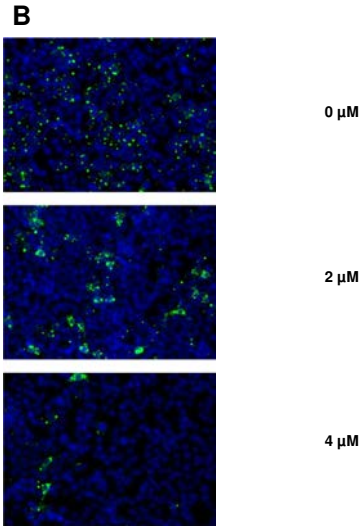
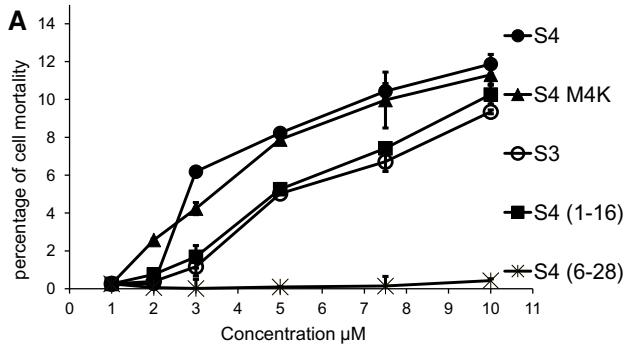


Fig. 1. Cytotoxicity and antiviral activity of dermaseptins. (A) The cell mortality due to dermaseptins was calculated as the percentage of lactate dehydrogenase (LDH) amount released in the extracellular medium by BSR cells treated for 2 h at the indicated concentration of dermaseptin compared to 100% released upon complete lysis of the cells. Dermaseptins S4, S4_{M4K}, S3, S4₁₋₁₆ and S4₆₋₂₈ were tested at concentrations ranging from 1 to 10 µM. Results are means of two independent experiments performed in duplicate. (B) BSR cells were infected with RABV PV strain (MOI = 1) for 24 h in presence of the dermaseptins (from 1 to 10 µM). The anti-RABV activity was assessed as the percentage of inhibition in comparison to mock-treated cells (100% infection). The results are means of three independent experiments performed in duplicate. (C) Infection of BSR cells with the RABV PV strain (MOI = 1) was performed in presence of different concentrations of dermaseptins S4. The infection was revealed using a rabbit polyclonal antibody directed against the RABV ribonucleoprotein conjugated to fluorescein.

any impairment in physiology or locomotion (data not shown). An additional group of control mice was challenged with the RABV CVS strain (22.5×10^5 UFF/mouse). This group demonstrated a sur-

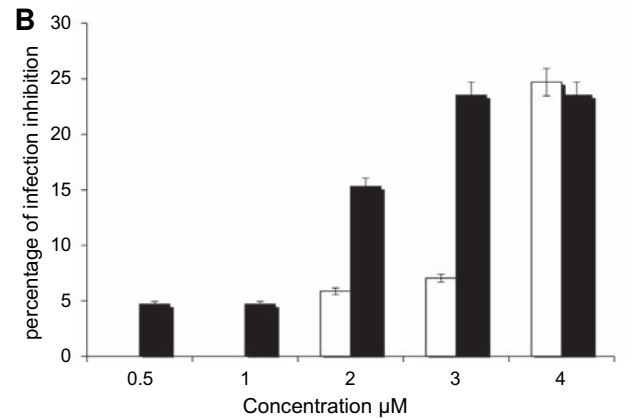
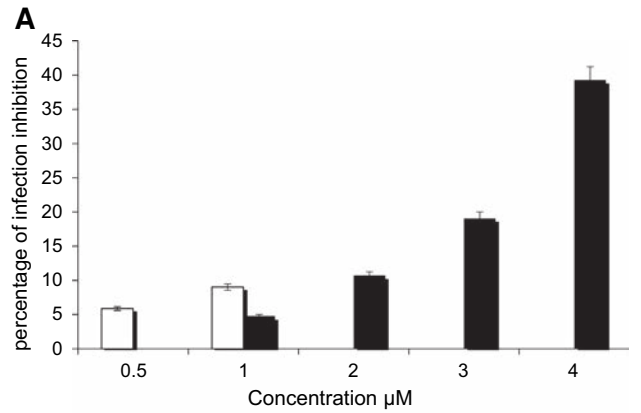


Fig. 2. Antiviral activity of the dermaseptin pre- and post-infection. The antiviral activity of dermaseptin S4 (A) and S4_{M4K} (B) was assessed when added for 1 h to culture medium, then removed from the supernatant before infection (white) or when added 1 h after (black) the infection of BSR cells with RABV (MOI = 1). The results are means of two independent experiments performed in duplicate.

vival rate of only 37.5% (Fig. 3). When 100 µg or 200 µg of the dermaseptin S4_{M4K} were inoculated 1 h after RABV infection at the same site, the survival rate was substantially increased to 62.5% and 75%, respectively. This corresponds to a survival difference of 25% and 37.5% compared to the control mice challenged with RABV. Surprisingly, inoculation of 100 µg or 200 µg of the dermaseptin S4 had no protective effect, and even resulted in a slight decrease in the survival rate to only 12.5% and 25% respectively. The mice which succumbed to clinical disease started to show clinical signs by day 6–9 post-infection and were humanely euthanized by day 8–11. There was no obvious difference in timing to death between the groups, suggesting that the antiviral effect of dermaseptins is most probably due to their RABV neutralizing potency at the early stages of infection.

We further explored the protective effect of 100 µg and 200 µg of dermaseptin S4_{M4K} in groups of 10 mice infected with different concentrations of RABV CVS strain: 9×10^5 UFF, 22.5×10^5 UFF and 45×10^5 UFF/animal. Without treatment with the dermaseptin S4_{M4K}, mice demonstrated survival rates of 50%, 37.5% and 0% respectively (data not shown). Again no mortality was observed in mice that received only dermaseptin S4_{M4K} (data not shown). Interestingly, a dose dependent protective effect was observed when dermaseptin S4_{M4K} was administered 1 h after RABV infection, at the same site. In comparison, 100 µg and 200 µg of S4_{M4K} were both able to induce 50% of survival gain in mice inoculated with 9×10^5 UFF of RABV (Fig. 4). The survival gain following administration of 100 µg of S4_{M4K} decreased when the viral dose increased to 22.5×10^5 UFF (40% gain) then 45×10^5 UFF

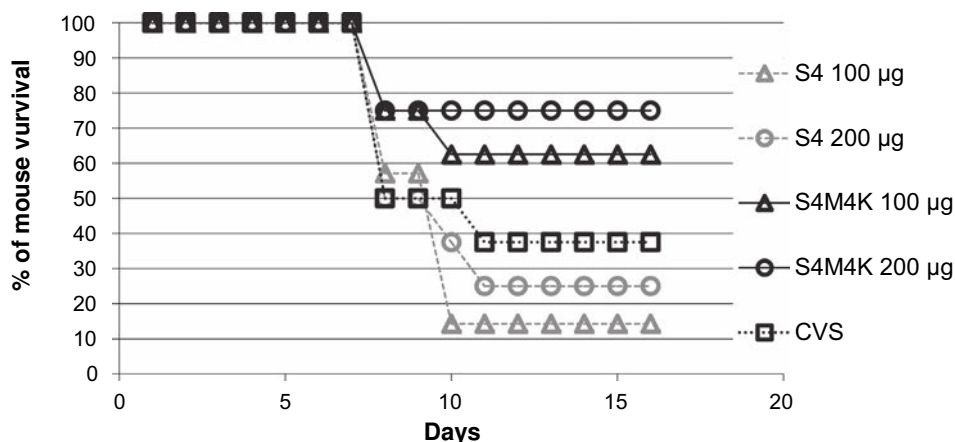


Fig. 3. Antiviral activity of dermaseptin S4 and S4_{M4K} *in vivo*. Experimental groups of 8 mice each were used to assess the antiviral activity of dermaseptin *in vivo*. One control group was inoculated in the gastrocnemian muscle with the RABV CVS strain (22.5×10^5 UFF per animal). Four other groups were inoculated identically but, 1 h after infection, they were again inoculated at the same place with 100 µg or 200 µg of dermaseptins S4 or S4_{M4K}. Mice were monitored for 16 days.

(20% gain). In contrast, a dose of 200 µg of S4_{M4K} maintained an improvement of 50–60% of survival gain with the three viral doses. This observation again suggests that the antiviral effect of dermaseptins is due to their RABV neutralizing potency, 200 µg being able to titrate higher viral loads.

4. Discussion

Antimicrobial peptides (AMPs) are secreted by many organisms including mammals, fishes, amphibians and insects to neutralize pathogens of their microenvironment. Dermaseptins isolated from skin frog display a particularly wide antimicrobial spectrum extending from eukaryotic parasites including protozoa and fungi, up to prokaryotic pathogens such as bacteria and viruses. If their antiviral activity originally targets the frog microenvironment, such as against frog virus 3 or channel catfish virus [32,45], it has the potential to be extended to human enveloped viruses including HSV-1 and 2, HIV-1 and HPV-16 [28,33–35]. In this context, the potential of dermaseptins against RABV infection has been investigated.

Dermaseptin S4 demonstrated a more efficient anti-rabies activity than dermaseptin S3. At a concentration of 7.5 µM, S4

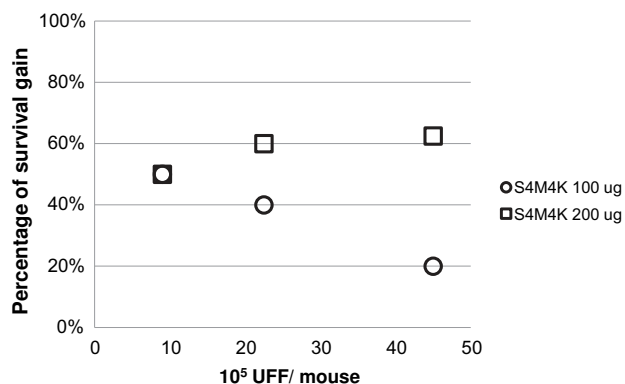


Fig. 4. Assessment of dermaseptin S4_{M4K} antiviral potential *in vivo*. Groups of 10 mice were inoculated in the gastrocnemian muscle with increasing doses of the RABV CVS strain: 9×10^5 UFF, 22.5×10^5 UFF and 45×10^5 UFF per animal. For each dose, 1 group was only infected while 2 others were injected 1 h later with 100 µg (circles) or 200 µg (squares) of dermaseptins S4_{M4K} at the same place of infection. Mouse survival was followed for 16 days. The graph presents the percent of survival gain due to S4_{M4K} injection compared to mice that received only RABV.

inhibited >85% of BSR cell infection by RABV compared to only 38% for S3. In contrast, it was previously shown that S3 is active against the intra-erythrocytic form of *Plasmodium falciparum* while S4 is not [39]. As S4 and S3 are 60% identical (Table 1), the aim was to identify the principal residues/regions supporting the anti-rabies effect. The S4/S3 similarity is spread into three main boxes: box 1 comprises the first three NH2 residues ALW, the tryptophan being the most conserved residue among the dermaseptins isolated from frog skin secretions; box 2 is restricted to residues LK in position 7–8; box 3 is more spread from position 12 to position 26 (for S4) and 28 (for S3) and not exactly conserved (3 mismatches and 2 gaps). Two deleted analogues of dermaseptin S4 were assessed: S4_{6–28} missing the five NH2 residues, including the conserved box 1, demonstrated no anti-rabies activity; S4_{1–16} conserving boxes 1 and 2 but missing most of box 3 retained a moderate, but still detectable, anti-rabies activity. This result clearly indicated that the specific anti-rabies activity of dermaseptin S4 is predominantly associated with the first five NH2 terminal amino-acids, although the COOH terminus plays an additional role, probably through stabilization of the interaction. Accordingly, the COOH terminus of dermaseptin S4 has been shown to enhance binding to target membranes [46]. In addition, a deletion analysis of dermaseptin B2 evidenced that the COOH end is necessary to allow a flexible helix-hinge-helix structure that facilitates the insertion of the peptide into the membrane [47].

As the first five NH2 residues of S3 and S4 only differ at position 4 and 5, position 4 of S4 (Met) was mutated in the corresponding residue in the S3 sequence (Lys), thus generating dermaseptin S4_{M4K}. This resulted in a very slight decrease of cytotoxicity in accordance with previous observations that substitution of a hydrophobic by a positively charged amino-acid reduced the hemolytic activity of dermaseptins and enhanced their selectivity for bacterial plasma membranes [23,48]. However, S4_{M4K} *in vitro*, retained similar anti-rabies activity to S4 when given at the time of cell infection: it inhibited approximately 50% of the infection at 5 µM and >85% above 7.5 µM. This is also in agreement with previous data demonstrating that position 4 of the dermaseptins is relatively permissive to mutations and can vary from basic to Threonine residues without notable effect on their biological activity [28]. Consequently, position 5 (Thr for S4, Asn for S3) could be crucial for the inhibitory activity, a hypothesis that might be further tested with dermaseptin S4_{T5N}.

Taken together, dermaseptin S4 (1–16) appears an appropriate candidate for construction of a novel peptide by introducing substitution or modification for the purpose of enhancing the selective

activity, while decreasing the cytotoxicity. Indeed, the acyl substitution of N-terminal sequence of a dermaseptin S4 derivative induced selective anti-pseudomonas activity with reduced toxicity towards human red blood cells [46,48–50].

Incubating BSR cells with dermaseptins 1h before the infection did not demonstrate a significant inhibitory effect (Fig. 2): while S4_{M4K}, logically acted in a dose dependent manner, S4 was weakly inhibitory at low concentration only, but had no effect at high concentration. It is well known that the concentration of dermaseptins is crucial for their specific activity [51–59]. This concentration is crucial to trigger self-association in four to six monomers and to facilitate channel insertion into membranes [47]. It may be speculated that pre-incubation at high concentration of dermaseptin S4 favored stable polymer channels on BSR cell membranes, leaving less monomers free to act on the virus membrane. At lower concentration, monomers of dermaseptin may remain more available to interfere with virus entry.

An interesting observation was that both S4 and S4_{M4K} were more efficient at inhibiting RABV infection when added 1 h after BSR cell infection. This suggests that beside their direct virucidal effect, dermaseptins may also affect the early stages of the intracellular infection, when the virus has been internalized or in the early stages of endocytosis. This result is in accordance with previous studies demonstrating that dermaseptins S3 and derivative of S4 selectively act against intracellular parasites [26,39,60].

Furthermore, the RABV inhibitory potential in mice of dermaseptins S4 and S4_{M4K} was assessed. Only dermaseptin S4_{M4K} induced a significant enhancement of the survival rate of infected mice, while dermaseptin S4 did not. Although no detectable toxic changes were observed in cells or tissues upon inoculation with dermaseptin to the mouse gastrocnemius muscle at the doses tested, as already reported with similar concentrations of antibiotics [58], it is not excluded that dermaseptin S4 may be slightly more toxic for animals, as it is *in vitro*. However, the antiviral activity of the dermaseptin S4_{M4K} appeared promising and functioned in a dose dependent manner: 100 µg and 200 µg, resulted in 62.5% and 75% of survival rate, respectively, compared to only 37.5% observed in mice that only received the virus. This dose dependent protective effect of dermaseptin S4_{M4K} was further confirmed against different viral loads, reaching 50–60% of survival gain compared to mock treated mice. This outlines its ability to titrate and neutralize RABV at the infection site.

5. Conclusion

In this study, the anti-rabies activity of dermaseptins S3 and S4 was assessed, and the residues involved in their antiviral effect were identified. *In vitro* kinetic experiments have shown that dermaseptins can act at the time of infection, but also post-infection, suggesting that their action is not limited to destabilization of the viral membrane but can also interfere with downstream steps of the virus cycle. *In vivo* studies have shown that S4_{M4K} is the more efficient dermaseptin, with 50–60% of survival gain in mice infected 1 h earlier with a lethal dose of RABV. In the context of RABV infection and PEP, such an early post-infection antiviral effect would be of paramount interest. Indeed, RABV transmission to human mostly occurs through dog (or other rabid animal) bite. The virus replicates locally at the site of bite before reaching the neuron endings and travelling towards the central nervous system through retrograde axoplasmic flow [61]. These early steps of infection are a crucial target for the anti-RABV strategy, and the World Health Organization recommends, in the case of a category III (severe) exposure, to complement post-exposure vaccination with extensive wound cleansing with soap of the area of bite, followed by a local instillation of anti-rabies immunoglobulins. The

latter being in dramatic shortage worldwide, a local application of active dermaseptins could substantially contribute to inactivation of the viral particle and possibly to act against the first steps of endocytosis.

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Contributors

MBM, AB, KH and NT have participated in the conception and design of the study. The experiments have been performed by MBM, CJ and KLM. These data were acquired, analyzed and interpreted by MBM, KLM, ARF and NT. The manuscript was drafted by MBM, GC and NT and was critically revised by AB, KLM, ARF and KH. All authors have approved the final submitted version.

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