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Evaluation of the antiviral activity of new dermaseptin analogs against Zika virus

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ABSTRACT

Zika virus represents the primary cause of infection during pregnancy and can lead to various neurological disorders such as microcephaly and Guillain-Barré syndrome affecting both children and adults. This infection is also associated with urological and nephrological problems. So far, evidence of mosquito-borne Zika virus infection has been reported in a total of 89 countries and territories. However, surveillance efforts primarily concentrate on outbreaks that this virus can cause, yet the measures implemented are typically limited. Currently, there are no specific treatments or vaccines designed for the prevention or treatment of Zika virus infection or its associated disease. The development of effective therapeutic agents presents an urgent need. Importantly, an alternative for advancing the discovery of new molecules could be dermaseptins, a family of antimicrobial peptides known for their potential antiviral properties. In this study, we carried out the synthesis of dermaseptins and their analogs and subsequently assessed the bioactivity tests against Zika virus (ZIKV PF13) of dermaseptins B2 and S4 and their derivatives. The cytotoxicity of these peptides was investigated on HMC3 cell line and HeLa cells by CellTiter-Glo® Luminescent Cell Viability Assay. Thereafter, we evaluated the antiviral activity caused by the action of our dermaseptins on the viral envelope using the Fluorescence Activated Cell Sorting (FACS). The cytotoxicity of our molecules was concentration-dependent at microgram concentrations Expect for dermaseptin B2 and its derivative which present no toxicity against HeLa and HMC3 cell lines. It was observed that all tested analogs from S4 family exhibited antiviral activity with low concentrations ranging from 3 to 12.5 μg/ml , unlike the native B2 and its derivative which increased the infectivity. Pre-incubating of dermaseptins with ZIKV PF13 before infection revealed that these derivatives inhibit the initial stages of virus infection. In summary, these results suggest that dermaseptins could serve as novel lead structures for the development of potent antiviral agents against Zika virus infections.

1. Introduction

Due to the ongoing geographic spread of both virus and its mosquito vector "*Aedes aegypti*", Zika virus (ZIKV) infections represent a significant global threat to public health. This virus is a member of the *Flavivirus* genus from *Flaviviridae* family. According to the World Health Organization (WHO), ZIKV infection has been correlated with an increased occurrence of neurological disorders, including Guillain-Barré syndrome and microcephaly [1]. In February of 2016, WHO announced a declaration of Public Health Emergency of International Concern (PHEIC) regarding these neurological complications [1]. A recent investigation conducted by Alcendor has revealed that the ZIKV is able to infect glomerular podocytes as well as renal glomerular endothelial and mesangial cells [2]. The transmission of ZIKV can be categorized into vector-borne and non-vector-borne modes. These two modes are distinguished by the presence of several species of *Aedes* mosquitoes in

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vector-borne transmission. Conversely, non-vector-borne human transmission occurs through transplacental transmission, blood transfusion or sexual transmission [3]. For over six decades, ZIKV infections have been characterized as mild, with approximately 80 % of cases either displaying no symptoms or experiencing short-lived and mild symptoms [4]. Since early 2007, epidemics of ZIKV disease have been reported not only in the Americas but also in Africa, Asia and the Pacific region [1]. During this epidemic, it was reported that approximately 73 % of Yap's 6892 residents aged 3 and older were affected by the ZIKV [5]. Subsequently, between 2013 and 2014, the virus spread to four other Pacific island groups, including French Polynesia, where it resulted in thousands of suspected infections and underwent in-depth investigations [1]. The clinical symptoms of patients primarily vary based on the population and the timing of the infection. In adults, the clinical manifestation is typically a self-limiting, acute, and febrile illness. It shares similarities with other infections caused by other arboviruses, such as dengue virus and Chikungunya virus. It differs from other viruses by several symptoms, including fever, prostration, retro-orbital pain, myalgia, and a maculopapular rash [6].

At present, there are no specific medications available to treat or prevent ZIKV infections. The current approach to treatment relies on administering fluids and ensuring infected patients get adequate rest. Painkillers, such as paracetamol, are generally used to relieve symptoms such as headache, fever and myalgia [7]. It is important to note that there is currently no vaccine available for the prevention or treatment of this infection. The development of a Zika vaccine continues to be an active subject of future research. The development of antiviral drugs against ZIKV has involved employing four approaches, which encompass targeting viral proteins, targeting host proteins, repurposing clinically approved drugs, and the use of reverse genetics systems such as clones and infectious cDNA replicons [7]. Numerous compounds have demonstrated to be effective against ZIKV *in vitro* assays, but to date; none have undergone assessment in clinical trials thus far [8]. Nonetheless, creating an effective and safe ZIKV vaccine poses different challenges. On the other hand, vaccines for other arthropod-borne flaviviruses, such as West Nile virus (WNV), dengue virus (DENV), tick-borne encephalitis virus (TBEV), yellow fever virus (YFV), and Japanese encephalitis virus (JEV), are presently accessible [3]. ZIKV stands out as distinctive since no other mosquito-borne virus has been identified as causing congenital disabilities and being transmitted sexually. A vaccine designed for one serotype of ZIKV could exacerbate infections from other serotypes or closely related viruses. This occurrence is referred to as antibody-dependent enhancement (ADE), which can occur when antibodies produced during an initial infection lack sufficient concentration or avidity to effectively neutralize a different serotype. Instead, these antibodies may lead to increased infectivity of target cells by facilitating virus opsonization and Fc-receptor-mediated endocytosis [3]. Moreover, the substantial reduction in Zika virus infection cases following the 2015–2016 epidemic posed challenges in conducting phase 3 trials, despite vaccine candidates successfully completing phases 1 and 2 trials [9].

Given these circumstances, there is an imperative demand for the development of novel drugs to combat ZIKV infection. Optimally, these drugs should demonstrate efficacy at low concentrations, exhibit non-toxic properties, and selectively target this enveloped virus. Cationic antimicrobial peptides (AMPs), particularly dermaseptins S4 and B2, could be considered as promising agents for combating this pathogen. AMPs are short peptides, generally consisting of 5–100 amino acid residues, and they exhibit a broad spectrum of molecular weights, typically below 10 kDa [10,11]. These peptides are generated by the innate immune systems of diverse organisms, including plants, insects, amphibians, and mammals. These peptides are originated from a variety of sources, including macrophages, neutrophils, epithelial cells, hemocytes, fat bodies, and reproductive tracts, among others [12]. Within this spectrum, one notable family of AMPs is dermaseptins (DRSs), which have been identified in the skin of the South American *Phyllomedusa* frog. DRSs are emitted by the granular glands located on the skin of amphibians, serving as a crucial element of their defense system against pathogens. Typically, these peptides comprise 28–34 amino acids, demonstrating significant diversity among distinct variants. However, in non-polar solvents, they have a tendency to adopt amphipathic α-helical structure [13]. DRSs demonstrate significant diversity in both their peptide sequences and lengths. Nonetheless, these cationic peptides share structural resemblances, including the presence of a conserved tryptophan residue at position 3, a conserved sequence of AA(G)KAALG (N)A in the middle region, and an overall positive charge [14]. In general, DRS-S peptides extracted from the secretions of the tree frog *Phyllomedusa sauvagii* have not been extensively studied for various human antimicrobial applications. These applications are particularly intriguing due to the peptides' ability to kill pathogens effectively without encountering resistance and their selective mechanism for microbe elimination [15]. To date, a total of thirteen DRSs (DRS-1 to DRS-13) have been identified and thoroughly characterized [16].

These peptides can function through different modes of action. Based on previous studies, the first proposed mechanism is known as the "barrel-stave" model, in which dermaseptins attach to membrane phospholipids. This attachment influences the osmotic balance of the host cell, resulting in membrane permeabilization. Thereafter, the formation of *trans*-membrane pores occurs, ultimately leading to the rupture of the pathogen membrane. The second commonly recognized mode, known as the "carpet-like" mechanism, involves the attachment of lytic peptides with a positive charge to a negatively charged surface. This destruction method facilitates complete surface coverage, leading to the infiltration and disintegration of the microbial membrane [17]. Some DRSs exhibit remarkable antimicrobial properties by effectively inhibiting the growth of microbial cells in a manner that doesn't induce toxicity to mammalian cells [15]. In addition to their broad spectrum of activity, DRSs generally do not cause hemolysis, except for DRS-S4, which exhibits significant hemolytic activity [16,18]. In a comparable context, DRS-B2, derived from the tree frog *Phyllomedusa bicolor*, is alternatively known as adenoregulin because of its ability to influence the attachment of adenosine A1 receptor agonists [19,20]. Among the DRSs family, DRS-B2 stands out by displaying the highest level of activity, which has led to extensive research focused on this particular peptide. This cationic polypeptide, characterized by amphipathicity and a positive charge (+3), consists of 33 amino acids. It has a molecular weight of around 3180 Da, with a tryptophan residue positioned at position 3 and six lysine residues [21]. While possessing an α-helical structure, this peptide demonstrates the capability to disrupt the membranes of various microorganisms, including bacteria, yeast, fungi, and protozoa. Nevertheless, its precise mechanism of action remains elusive [22].

To our knowledge, there have been no studies conducted to assess the antiviral effects of the native molecules S4 and B2, as well as their derivatives, against the Zika virus. Therefore, in this present study, we are presenting, for the first time, the *in vitro* antiviral efficacy of these peptides against the Zika virus. DRS derivatives were synthesized, purified, and evaluated for their antiviral activity. Their cytotoxicity toward HeLa and HMC3 cells was evaluated, and then Fluorescence Activated Cell Sorting (FACS) was performed to evaluate the infected and viable cell populations.

2. Material and methods

2.1. Conception and purification of analogs of dermaseptins

Using a Milligen 9050 pepsynthesizer, stepwise solid phase synthesis utilizing Fmoc polyamide-active ester chemistry was used to synthesize the peptides. The source of all Fmoc-amino acids was Milligen/Bioresearch–Waters, based in Paris, France. The substances 4-(Hydroxymethyl) phenoacetic ac-id-linked polyamide/kieselguhr resin (pepsin kA), Fmoc-aminoacidpentafluorophenyl (Pfp), and 3-hydroxy-2,3-dehydro-4-oxo-benzotriazine (Dhbt) esters were obtained from Milligen/

Bioresearch (Paris, France). Using 5 mg of peptidyl-resin in 1 mL of a solution consisting of trifluoroacetic acid, paracresol, thioanisol, water, and ethyl methyl sulfide (82.5 %, 5.5 %, and 2.5 % (v/v)), side chain deprotection and pep-tidyl-resin cleavage were performed for 2 h at room temperature. The crude peptides were purified using a mixture of preparative high performance liquid chromatography (HPLC), ion exchange chromatography, and Sephadex gel filtration following filtering to remove the resin and ether extraction. Mass spectrometry, solid phase sequence analysis, analytical HPLC, and amino acid analysis were used to evaluate the homogeneity of the synthetic peptides [13]. Each peptide was maintained frozen as stock solutions in double-distilled water at −20 °C, with a final concentration of 3.5 mM.

2.2. Evaluation of the physicochemical and structural characteristics of dermaseptins

The BACHEM peptide calculator tool was used to determine the length, molecular weight (MW), and net charge (Z) of our peptides. Using Heliquest software, hydrophobicity (H) and hydrophobic moment (μH) were determined [23]. TANGO software 2.2 was used to calculate the overall tendency for aggregation in an aqueous solution [24], and AGADIR software 2s was used to predict the helicity (α-helix%) of each peptide [25].

2.3. Cell culture

The HMC3 and HeLa cells are cultured in an incubator in the presence of high humidity and 5 % CO_2. Cells were cultured in Dulbecco's Modified Eagle Medium DMEM (+L-Glutamine) which was supplemented with 10 % FCS 50 ml (Calf Serum Fetal) and 1 % antibiotics (Penicillin/Streptomycin). The cells were routinely seeded three times a week. Cell enumeration was conducted using an automated TC20 cell counter.

2.4. Cell viability test: CellTiter-Glo

The HMC3 and HeLa cells are seeded in 96-well plates. Each well must contain 10^4 cells/100 μl in the end. ½ dilutions of the different peptides were previously prepared in eppendorf tubes. These concentrations range from 100 μg/ml to 1.5 μg/ml 50 μl of each peptide were added to the plate and the tests are carried out in triplicate. After 2 h of incubation at 37 °C and 5 % CO2; the medium was aspirated and replaced with 100 μl of DMEM medium containing 10 % FCS. The cells were then incubated at 37 °C at 5 % CO_2 for 24 h to test cell viability. These tests were performed in the presence of a negative control (cells without peptide). A volume of CellTiter-Glo reagent equal to the volume of cell culture medium present in each well is added. In our case, the volume of reagent added is equal to 100 μl. Then we mixed the contents of the plate for 2 min on an orbital agitator to induce cell lysis. After 10 min of incubation necessary for the stability of the luminant signal, the luminescence is recorded using the plate reader with the software Skanit RE 4.1. In this method, the quantity of viable cells in culture is determined by measuring the ATP content. ATP acts as a marker for metabolically active cells. Upon cell lysis, ATP is released and engages in a luciferin/luciferase reaction.

2.5. Viral strain and ZIKV titration

The viral strain used in our study was generously provided by the "Genomics and Vaccination" department at the Institute Pasteur in Paris. This strain known as PF13, was initially isolated from French Polynesia in 2013. It's worth mentioning that French Polynesia encountered its most notable epidemic between October 2013 and April 2014, as documented by Cao-Lormeau et al. [26].

The titration technique is applicable to all viruses that induce a distinctive cytopathic effect. The assessment of viral titer is determined using a statistical formula based on the number of inoculated wells. In flat-bottomed 24-well microtiter plates, we conducted the titration process: 10^5 HMC3 cells were added per well along with 500 μl of DMEM containing 5 % FCS. On day 1, our cells were expected to reach 90–95 % confluence, and we prepared the CMC salt (Carboxymethylcellulose stored at 4 °C) at room temperature. The cells were then washed twice with DMEM containing 0 % FCS (Fetal Calf Serum). Thereafter, we added 200 μl of 1 ml DMEM 2 % FCS for the quadrulicates, homogenizing by pipetting 10 times. Then, 200 μl of viral dilution/well was added, starting with the negative control (DMEM, 2 % FCS) and proceeding to higher dilutions. Subsequently, we incubated the plates for 2 h at 37 °C. Once the CMC and DMEM with 5 % FCS were thoroughly mixed, we prepared a 20 ml solution for a 24-well plate. After a 2-h infection period, the inoculum was extracted from each well, including those from the uninfected and highest dilutions. Afterward, 400 μl of 2 % DMEM was gently added using combitips, followed by the addition of 400 μl of CMC/medium. The plate was incubated for 4 days at 37 °C. Then, on the 4th day, two washes were carried out with PBS. Subsequently, 400 μl of crystal violet solution was added and incubated for 20 min at room temperature, followed by rinsing with water. Finally, the count of plaques for each dilution was determined, and the average per well was calculated according to this formula: **Nb PFU/ml = number of plate X5 (convert 200 μl on 1 ml) X dilution factor.**

2.6. Treatment of ZIKV PF13 with dermaseptins

The HMC3 cells were first seeded in 24-well plates at a concentration of 4.10^4 cells/well on day 0 in order to treat the virus with our different peptides. On the following day, we proceeded with our four peptides, B2, $K_4K_{20}K_{27}S4$, $K_4S4(1–16)$, and $K_4K_{20}S4$, at ½ dilutions. 10 μl of ZIKV PF13 at a multiplicity of infection (MOI) of 1.5 was added to each dilution. The incubation was carried out at 37 °C for 1 h. These tests were conducted in duplicate. In virology, the term "Multiplicity of infection" (MOI) is frequently used to describe the number of viral genomes that contribute to the infection of each cell throughout the regular process of host invasion. It is calculated using the formula that outlined below: **MOI= Zika PFU (plaque forming units)/number of cells.**

Subsequently, 200 μl of the Mix (peptide + virus) was added to each well of the cells, and the mixture was incubated for 1 h and a half at 37 °C. The medium was aspirated and replaced with fresh culture medium following the incubation time. This prepared mixture will be used to perform FACS analysis.

2.7. Evaluation of inhibition of CHME3 cell infection by FACS technique

The cells were first distributed into 24-well plates. Subsequently, we detached the cells using trypsinization and transferred them to eppendorf tubes. Then, the cells were fixed using the cytofix/cytoperm reagent and incubated for 30 min at 4 °C. Two washes were performed, followed by a centrifugation at 400*g* for 5 min at 4 °C. This step is conducted to enable the removal of the supernatant fluid with minimal loss of cells. For indirect labeling, a two-stage incubation process was employed for 1 h at 4 °C in the dark. Initially, the cells were incubated with a 4G2 primary antibody, which is specific to the Flavivirus envelope. This was followed by a 1/500 dilution in perm/wash 1X, with 100 μl per tube. Afterward, the cells were washed twice by centrifugation with 500 μl of perm/wash 1X each time. Later, they were incubated with a compatible secondary antibody.

The secondary antibody used has the fluorescent dye Alexa488 conjugated. A dilution of 1/1000 (100 μl/tube) was prepared, followed by two washes of the cells through centrifugation at 400*g* for 5 min. Finally, the cells were suspended in PBS (1000 μl) for analysis on the flow cytometer using the Flowjo-v10 software.

3. Results and discussion

3.1. Conception and synthesis of analogs of dermaseptin S4 and B2

The peptides in our study were generated by a succession of structural modifications, mainly including deletions and/or substitutions in the S4 and B2 regions (Table 1). By substituting lysine (K), designated as M4K and N20K, respectively, for methionine (M) at position 4 and asparagine at position 20, the derivative $K_4K_{20}S4$ was produced. Methionine was also substituted at position 27 in the second derivative, $K_4K_{20}K_{27}S4$, in an attempt to further enhance its cationicity. The M4K alteration is also present in the peptide $K_4S4(1–16)$, however 12 C-terminal residues have been deleted. K_3K_4B2 was produced by means of double substitution, wherein lysine (K) was substituted for a tryptophan residue (W) at position 3 (W3K) and lysine (S) was substituted for a serine residue (S) at position 4 (S4K) of B2. The amino-acid sequence of these peptides was represented by a one-letter code, with the length and sequence being compared to the natural peptides DRS-S4 and DRS-B2 (Table 1).

Reducing hydrophobicity and increasing hydrophilicity was the primary objective of the first modification phase. Our primary objective in altering this peptide was to increase its hydrophilic properties by adding basic amino acids, notably lysine, as S4 and B2 showed substantial impacts on functional activity related with cationic residues. A further consideration in the selection of lysine was to prevent any rise in cytotoxicity. Prior research revealed that despite maintaining strong biological activity, DRS-S4's hemolytic activity was decreased when its net positive charge was increased and its hydrophobicity was decreased [18,27,28]. It is possible to shorten S4's native sequence by removing 12 amino acids from its secondary structure [29].

Analog peptides have been designed using a variety of techniques to address these issues. These strategies include de novo design, which aims to reduce peptide length and eliminate host defense immunogenicity [32], truncation/substitution, which reduces toxicity [31], and motif hybridization, which aims to improve antimicrobial potential and functioning [30]. Previous research revealed that DRS's C-terminal domain largely exhibits nonspecific membrane lytic activity, while the N-terminal domain of the protein interacts with the bacterial cell membrane in a selective manner [33,34]. Research conducted previously on the N-terminal peptide extremities of DRSs showed that shortened peptides consisting of 16–19 amino acids retain comparable levels of antibacterial action. It is noteworthy that shorter sequences especially those with less than 13 amino acid residues show an apparent decrease in antibacterial effectiveness [35,36]. To summarize, the process of determining the structural prerequisites for biological activity includes investigating the peptide criteria for selection. As for B2, as far as we are aware, this research is the initial instance in which the derivative K_3K_4B2 has been used in an antiviral test. As a matter of fact, about all of the modifications performed to the original B2 molecule included truncation, resulting in the synthesis of a C-terminally truncated analog known as [1–23]-DRS-B2.

This shortened derivative exhibited no activity against bacteria while preserving the net cationic charge of the native peptide B2 [22]. When DRS-B2 and alginate nanoparticles (Alg NPs) are combined, a formulation known as Alg NPs + DRS-B2 is produced. This formulation produces novel B2 derivatives that have significant antibacterial activity against strains of *Escherichia coli* that are both susceptible and resistant to colistin. When used on its own, the antibacterial activity of this novel formulation is significantly higher compared to that of DRS-B2 [21].

Our synthetic peptides also have the added advantage of having D-amino acid structure. However, as L-amino acids are susceptible to degradation by proteases, peptides in their natural state represent a challenge that limits their application in clinical settings [37]. As previous studies have demonstrated, one practicable approach to overcome these restrictions is to substitute the L-amino acids in the most sensitive region with D-amino acids [38]. In fact, substituting D-amino acids maintains the native peptide's net positive charge; nevertheless, it does affect the structure and function associated with chiral target recognition [39,40].

3.2. Evaluation of physicochemical properties of dermaseptins S4 and B2

The $K_4K_{20}S4$ and $K_4K_{20}K_{27}S4$ peptides among the S4 derivatives were found to have the highest hydrophobicity values (0.451 and 0.437, respectively) based on Heliquest analysis, while $K_4S4(1–16)$ had the lowest value (0.426) (Table 1). All of the DRS-S4 peptide derivatives typically displayed hydrophobicity (H) values that were lower than those of the initial peptide (S4). This finding was also confirmed for DRS-B2 and its derivative, since the derivative's hydrophobicity (H) value was greater at 0.199 than K_3K_4B2's value of 0.072. The hydrophobicity of peptides is a crucial physicochemical parameter. Typically, peptide sequence analysis is used to estimate hydrophobicity [41]. This parameter is essential for controlling conformational changes, maintaining stability, and facilitating peptide molecular interactions [42]. The values of μH ranged from 0.159 to 0.526, where $K_4S4(1–16)$ showed the highest value (μH = 0.526) and K_3K_4B2 the lowest (μH = 0.159) (Table 1). The vector sum of the hydrophobicity values assigned to each individual amino acid in the peptide sequence is calculated to determine the hydrophobic moment (μH) [23,43]. The interfacial interaction between peptides and the cell membrane is significantly influenced by this characteristic [44]. Based on the TANGO algorithm [24], it was found that DRS-S4 had a higher tendency towards aggregation as opposed to its derivatives. The presence of two hydrophobic domains—one at the N-terminal and the other at the C-terminal—was suggested to be the reason of this tendency ((Table 1), (Fig. 1)). These results are consistent with those of Feder et al., who showed that hydrophobic interactions induce aggregation in DRS-S4 and its analogs. In particular, the aggregation propensity of K4S4 was decreased by the substitution M4KN20K in both the N-terminal and C-terminal domains, which is in accordance with findings from earlier studies [18,45]. However, aggregation was reduced as a result of the shortening of S4's C-terminal domain and the incorporation of positive charges in these regions. The aggregate value of $K_4S4(1–16)$ equaled zero, but $K_4K_{20}K_{27}S4$ displayed a value of 37.96 (Table 1). The tendency to aggregation will therefore probably be

Table 1
The physicochemical characteristics and sequences of dermaseptins and their analogs.

Peptides	Sequence[a]	Parameters[b]						
		Length	MW	Net Charge	H	Aggregation	μH	α-Helix %
S4 (Native)	ALWMTLLKKVLKAAAKAALNAVLVGANA	28	2.850	+4	0.544	183.33	0.248	16.55
$K_4K_{20}S4$	ALWKTLLKKVLKAAAKAALKAVLVGANA	28	2.861	+6	0.451	112.02	0.246	11.8
$K_4K_{20}K_{27}S4$	ALWKTLLKKVLKAAAKAALKAVLVGAKA	28	2.874	+7	0.437	37.96	0.254	13.11
$K_4S4(1–16)$	ALWKTLLKKVLKAAAK	16	1.782	+5	0.426	0	0.526	2.41
B2 (Native)	GLWSKIKEVGKEAAKAAAKAAGKAALGAVSEAV	33	3.181	+3	0.199	9.681	0.204	10.02
K_3K_4B2	GLKKKIKEVGKEAAKAAAKAAGKAALGAVSEAV	33	3.164	+5	0.072	9.681	0.159	9.85

[a] The sequences are shown using the one letter code for the amino acids.
[b] Parameters: MW (kDa); H: Hydrophobicity; Aggregation: total trend of aggregation; μH: Hydrophobic moment; α-Helix %: Helicity.

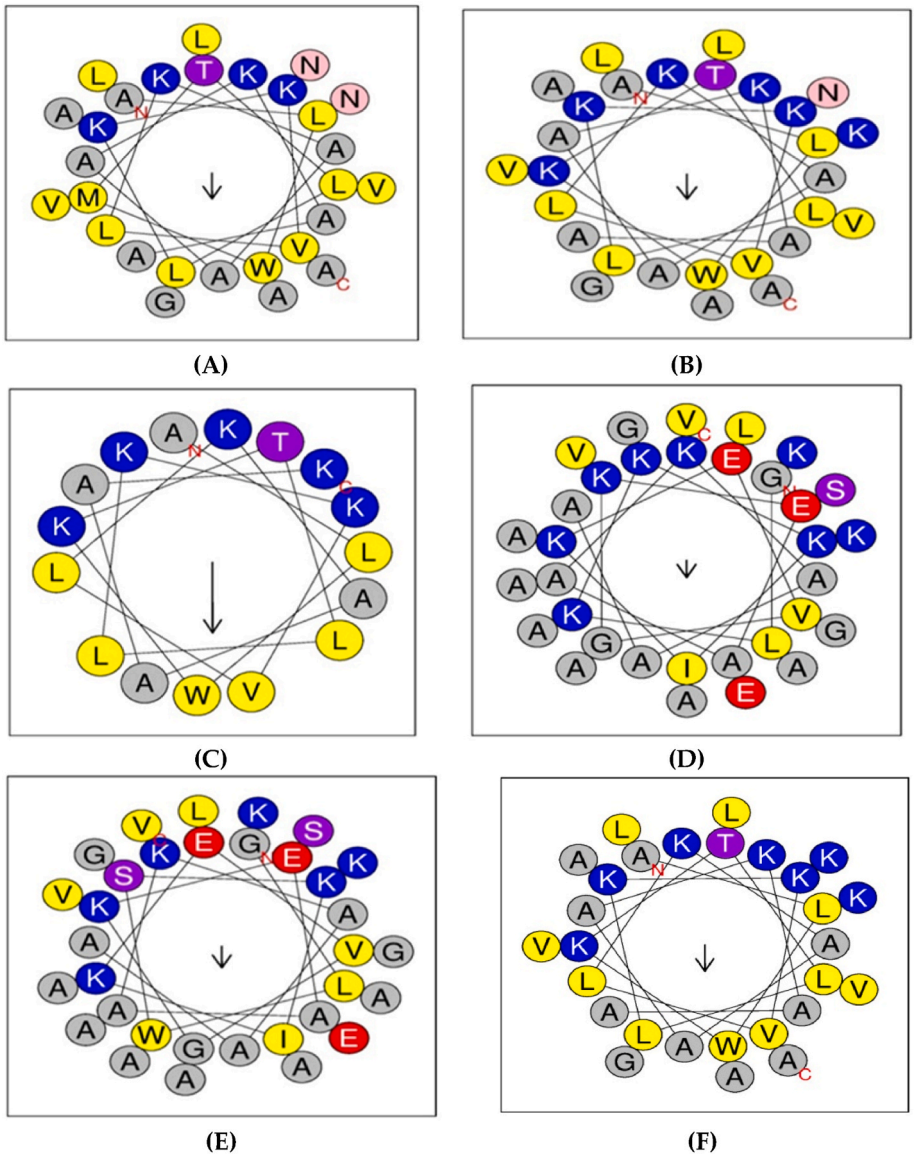

Fig. 1. Dermaseptin B2 and S4 Helical Structures and Their analogs. These peptides were represented as the 2-dimensional axial projection of an ideal α-helix. A) Helical structure of the native S4; B) Helix structure of K_4K_{20}S4; C) Helical structure of K_4S4(1–16); D) Helical structure of K_3K_4 B2; E) Helical structure of the native B2; F) Helical structure of $K_4K_{20}K_{27}$S4. The amino acids are presented with different colors according to their proprieties: (grey: nonpolar residue; blue: positively charged residue; yellow: hydrophobic residue; red: for acidic; pink: for Asn (N); purple: The Thr (T) and Ser (S); the arrow in helical wheels corresponds to the hydrophobic moment μH. Figure built using Heliquest software ComputParams form version 3 (Gautier et al., 2008). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

minimized by eliminating the hydrophobic domains and/or incorporating positive charges in these regions. Electrostatic repulsion between cationic residues or the lack of hydrophobic domains are the two possible causes of this [18,46]. These results show that charge distribution of peptides also influences their aggregation, in along with hydrophobicity. Consequently, aggregation is negatively impacted by adding cationic amino acids to one of the hydrophobic domains or by eliminating hydrophobic domains. It was shown that in this particular instance of the native DRS-B2, aggregation was not affected by substitution or deletion. This is noteworthy because, in contrast to S4, the native molecule already showed extremely low levels of aggregation (9.681) (Table 1). Prior research has shown that another important factor affecting antibacterial activity and cell selectivity is the tendency for aggregation in aqueous solution [47]. The peptides' capacity to aggregate (form oligomers) in aqueous solution, where they interact hydrophobically with other monomers, provides an explanation for this property. Such aggregates become unable to penetrate through bacterial cell walls, outer membranes, or polysaccharide capsules due to their increased size and decreased flexibility. As a result, they cannot interface with the plasma membrane, which is their designated site of action. In consequence, peptides that are very susceptible to aggregate usually have little to no impact on bacteria. In contrast, monomeric peptides have a greater ability to penetrate the plasma membrane, which allows them to have strong antibacterial properties [48]. For this reason, minimizing aggregation is usually preferred when creating antimicrobial peptides in order to increase antibacterial activity [28,48]. We found that all of our peptide derivatives showed less aggregation during our analysis, which might represent biological advantages for them, such as antiviral properties.

In order to evaluate the helicity of the peptides under investigation, we utilized the AGADIR method [25]. As for helicity (measured in α-helix%), the results revealed that the natural molecule S4 had the highest, around eight times higher than K4S4(1–16) and similar to K4K20S4. But DRS-B2 and its derivative showed a comparable helicity,

measuring 10.2 % and 9.85 %, respectively (Table 1). According to literature review [49], helicity measurement is used to investigate the relationship between secondary structure and selectivity against microbial cells, especially for α-helical antimicrobial peptides. Consequently, the preservation of the helical structure as well as a greater net positive charge may be associated with dermaseptins' increased potency [50].

3.3. Cytotoxicity of dermaseptins and their derivatives against HMC3 and HeLa cells

An antiviral drug should exhibit effectiveness without causing notable toxicity to host cells. To assess the cytotoxic effects of dermaseptins S4 and B2, along with their derivatives, we initially used the cellTiter-Glo Test to measure luminescence. Each data point represents an average obtained from triplicate assays. This test evaluates the viability of cells by quantifying the ATP levels present. The cell viability test was conducted on both HeLa and HMC3 cells. The HMC3 cells were subjected to varying concentrations of these peptides, ranging from 0 μg/ml to 100 μg/ml. It is important to highlight that the selection of these cells was determined by the availability of cell lines in the laboratory, and previous findings indicated that these cells are optimal for cultivating the Zika virus. The corresponding results are presented in the following figures ((Fig. 2) and (Fig. 3)).

According to Fig. 2, Dermaseptin B2 and its derivative K_3K_4B2 demonstrated no cytotoxic effects even at significantly high concentrations of approximately 100 μg/ml. However, for dermaseptin S4 derivatives, namely $K_4K_{20}S4$, $K_4K_{20}K_{27}S4$, and $K_4S4(1–16)$, while they exhibited cytotoxicity at 100 μg/ml, they remained non-toxic at concentrations of about 12.5 μg/ml. At concentrations below these values, the cells maintain viability. Remarkably, our findings indicate that the $K_4S4(1–16)$ derivative is the least toxic among the DRS-S4 derivatives, even at a concentration as high as 12.5 μg/ml when compared to other peptides. The findings observed on Fig. 3, illustrated that the dermaseptin B2 derivative, K_3K_4B2, remains non-cytotoxic even for the second HeLa cell line, even at high concentrations.

On the other hand, the S4 derivatives, which are $K_4K_{20}K_{27}S4$ and K_4S4 (1–16), exhibit cytotoxicity at concentrations of 50 μg/ml. Notably, $K_4K_{20}S4$ becomes cytotoxic at a lower concentration, around 25 μg/ml, making it the most toxic among the S4 derivatives, with a viability of approximately 1 % at 100 μg/ml. Additionally, $K_4K_{20}K_{27}S4$ is the least toxic among the three S4 derivatives, displaying a viability of 18 % at the same concentration.

The cytotoxicity of DRSs exhibited a concentration-dependent pattern, with peptide 50 % cytotoxic concentration (CC_{50}) values determined accordingly. Moreover, molecules with the lowest CC_{50} values are correlated with the least toxicity. The specific CC_{50} values for each are detailed in the following table (Table 2).

Regarding the interaction of our peptides with the cell types used in this test, it's noteworthy that HeLa cells displayed considerably higher

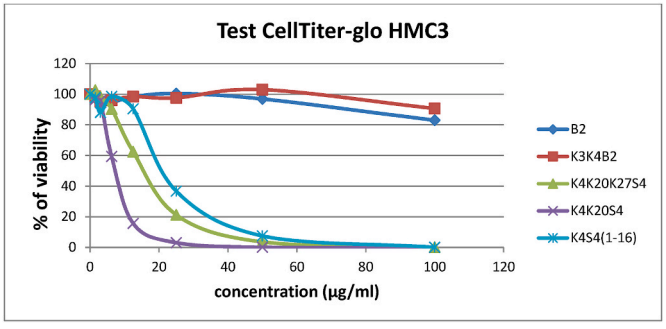

Fig. 2. Viability of HMC3 cells incubated with different concentrations of dermaseptins.

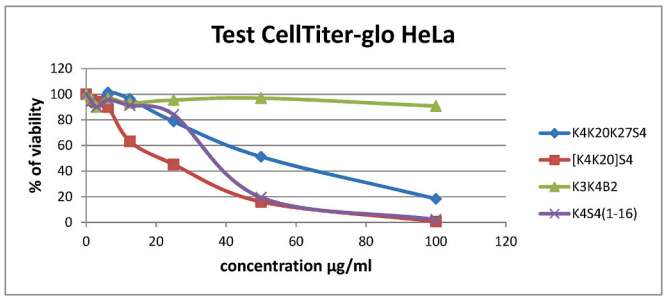

Fig. 3. Viability of HeLa cells incubated with different concentrations of dermaseptins.

Table 2
The values of CC_{50} on HeLa and HMC3 cell lines.

Peptides	CC_{50} HeLa	CC_{50} HMC3
$K_4K_{20}K_{27}S4$	59.18	33.60
$K_4K_{20}S4$	37.39	20.89
B2	ND	ND
K_3K_4B2	ND	ND
K4S4 (1–16)	45.71	37.30

CC_{50}: Concentration of the peptide (μg/ml) which causes 50 % cytotoxicity of cells. **ND:** not determined.

resistance to our peptides compared to HMC3 cells. For instance, at a concentration of 25 μg/ml, HeLa cells exhibited 45 % viability in contrast to only 3 % viability observed in HMC3 cells. Interestingly, our findings revealed that shortening the peptide at the C-terminal extremity of $K_4S4(1–16)$ led to a CC_{50} of about 45.71 μg/ml for HeLa cells and a lower value of approximately 37.30 μg/mL for HMC3 cells. These results confirm our hypothesis of the resistance of HeLa cells to the toxicity induced by high concentrations of our synthetic peptides. Moreover, increasing their positive charge through different substitutions ($K_4K_{20}S4$ or $K_4K_{20}K_{27}S4$), resulted in peptides with low toxicity displaying CC_{50} values of about 37.39 μg/ml and 59.18 μg/ml, respectively, for the HeLa cell line. This affirms that the introduction of a positive charge enhances the safety of our peptides for host cells toward toxicity. These results are also consistent in the case of HMC3, where the CC_{50} of the tri-substituted peptide is higher than that of the bi-substituted one (33.60 μg/ml and 20.89 μg/ml, respectively). To summarize, the highest cytotoxicity values for all S4 derivatives were recorded at concentrations above 37.30 μg/ml. However, the relationship between the concentration of peptide likely to cause toxicity to cells and the effective antimicrobial concentration is generally less than 100 *in vitro*. This selectivity can be optimized by biochemical modifications made to the peptide sequence [18].

The cytotoxicity of dermaseptin and its derivatives has been assessed in numerous prior studies involving various types of cell lines to confirm their safety for future clinical applications. Expect for the native S4 which shows toxicity towards mammalian cells and displays potent hemolytic effect [18]. A study carried out by Belaid et al. [51] indicated that the highest non-cytotoxic concentrations for DRS-S1 and DRS-S2 were 32 μg/mL, for DRS-S3 and DRS-S4 were 16 μg/mL, and for dermaseptin S5 were 64 μg/mL when assessed on HEp-2 cells. In a supplementary study conducted by Hazime et al. [21], the cytotoxic effects of DRS-B2 and a novel formulation (Alg NPs + DRS-B2) were examined on human erythrocytes and eukaryotic cell lines HT29 (human) and IPEC-1 (animal). In this investigation, the safety of these compounds was confirmed. Zairi et al. [15] confirmed that dermaseptin K_4S4 demonstrated a higher toxicity profile against human endometrial epithelial cells, indicating a reduced susceptibility to the toxic effects of dermaseptins when compared to other cell types. Although dermaseptin S4 and its derivatives have shown notable cytotoxicity against the SW620 cell

line, understanding their cellular selectivity or mode of action remains challenging [52]. Furthermore, Lorin et al. [53] illustrated that dermaseptin $K_4S4(1–16)a$ exhibited a similar effect on both HeLa P4-CCR5 cells and primary PBMCs. This peptide showed no toxicity in mice, and displayed reduced cell toxicity even at high concentrations. All of this research has indicated that the toxicity of peptides is highly dependent on the cell type. The synthesized and modified peptides were found to be less toxic to HeLa cells in comparison to HMC3 cells, which demonstrated greater susceptibility to our modified derivatives.

3.4. The anti-ZIKV activity of DRSs

The only strategy used when studying the antiviral effects of our peptides is, their *in vitro* evaluation against infected cells. In our work, we proposed to test for the first time dermaseptins as potential inhibitors of the Zika virus on microglia. The study of dermaseptins activity against the ZIKV PF13 strain was exclusively carried out on HMC3 cells. This choice is justified by the cells' increased affinity for the virus, as the infection primarily targets the central nervous system. Throughout this experiment, only non-cytotoxic concentrations of dermaseptins, where cell viability is greater than 90 % were tested. The concentrations chosen for each peptide are ranged from 50 μg/ml to 3 μg/ml, following a series of 1/2 dilutions. During this step, the peptide was introduced at an early stage of the viral cycle. In fact, we treated the virus with our peptides before infecting the HMC3 cells. This choice was made based on previous studies carried out on dermaseptins. Indeed, Lorin et al. have demonstrated that antiviral activity reaches its maximal activity during the first stage of the HIV viral cycle [53]. This finding was further confirmed by Bergaoui et al. through his research on the herpes virus (HSV-2) [54]. Fig. 4 showed that dermaseptins differentially inhibit ZIKV PF13 virus infection. In fact, only S4 derivatives exhibit anti-Zika activity. Approximately 90 % reduction in infection was observed in the presence of 6.25 μg/ml of $K_4K_{20}K_{27}S4$, 3 μg/ml of $K_4K_{20}S4$ and around 80 % for K_4S4 (1–16) at 12.5 μg/ml. A deletion carried out on the molecule shows that the C-terminal end is essential for the antiviral activity of the peptide [54]. Shortening the K_4S4 (1–16) peptide at the C-terminal region significantly reduced its anti-Zika activity, but impressively reduced its toxicity. Nonetheless, earlier research has indicated that $K_4S4(1–16)$ presented a considerable antimicrobial efficacy *in vivo*, without encountering any toxicity concerns even at elevated concentrations [55]. Based on the results of this study, it is reasonable to conclude that the C-terminal end plays a crucial role in anti-Zika activity. In contrast, dermaseptin B2 does not exhibit any antiviral activity; in fact, it demonstrated an increase in infection at a concentration of 50 μg/ml. These results provide evidence that three of our dermaseptins studied had potent antiviral activities. It was demonstrated that dermaseptin and its analogs were able to decrease the percentage of infection by ZIKV, with the exception of B2 and K_3K_4B2, which actually increased the infection. It's noteworthy that these peptides did not exhibit any toxic effects on HMC3 cells or HeLa cells. Our results revealed that increasing the positive charge of the B2 to synthesize the K_3K_4B2, and consequently enhancing its cationic nature, resulted in a loss of activity in the peptide. Surprisingly, this alteration not only led to a loss of its activity, but it also contributed to an increase in the percentage of cell infection by the virus. However, it is difficult to predict the cause of this increase as no other study relating to the antiviral activity of B2 derivatives has been carried out previously against the Zika virus to better understand this phenomenon. This loss of antimicrobial

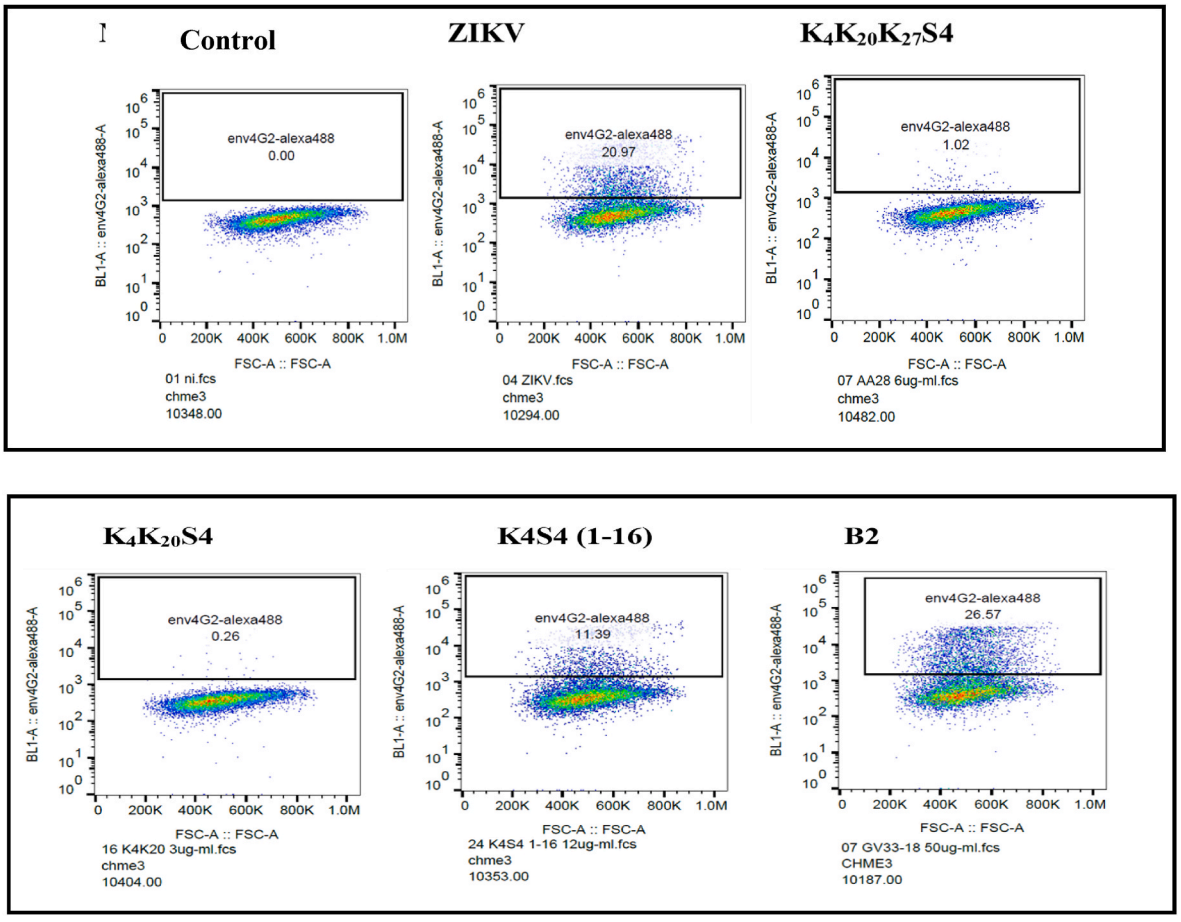

Fig. 4. Antiviral activity of dermaseptins on HMC3 cells. Infected cells are marked with the 4G2 anti-envelope antibody. Incubation of the virus with dermaseptin derivatives before infection. Peptides were added in different concentrations ranging from 50 to 3 μg/ml.

activity may be attributed in part to excessively strong interactions which would prevent the translocation of the peptide. This could potentially elucidate the inactivity of K_3K_4B2 despite its non-cytotoxic nature. The increase in cationic character is therefore not a determining and essential factor for the bioactivity of dermaseptins as long as K_3K_4B2 remains inactive.

The evaluation of the half-maximal (50 %) inhibitory concentration (IC_{50}) is essential to better understand the pharmacological and biological characteristics and perspectives of an antimicrobial molecule. The Selectivity index (IS) was calculated from the CC_{50} values from the cytotoxicity test carried out on HMC3 cells previously described (Table 3).

According to Table 3, dermaseptin $K_4K_{20}S4$ has the highest IS among the S4 derivatives (IS = 49.73) and K_4S4 (1–16) has the lowest selectivity index (IS = 1.71). We can therefore confirm that the substitution of one or more uncharged amino acids to positively charged amino acids, without shortening its sequence, reduces cytotoxicity without affecting anti-Zika activity. We can give an order for the activity potential of dermaseptins, it can be the following: $K_4K_{20}S4 > K_4K_{20}K_{27}S4 > K_4S4(1–16)$. It should be noted that the tri-substituted peptide $K_4K_{20}K_{27}S4$ of dermaseptin is tested for the first time for its antiviral activity. No previous studies have reported antiviral activities relating to it.

Our study showed that pre-incubation of dermaseptins with virus before infection resulted in strong inhibition of ZIKV infection at a concentration equal to 3 μg/ml. This suggests that dermaseptins act during the early stages of the viral cycle. Therefore, we can propose that its activity is aimed directly at the viral particle, potentially inducing lysis of the viral membrane. This lysis could result in the exposure and subsequent dissociation of the virus core. Indeed, considering that the Zika virus is enveloped, the peptide could interact with the phospholipids constituting the viral membrane as well as with the cellular receptors essential for virus entry. Moreover, the cationic character of the peptide facilitates electrostatic interactions between it and the phospholipids.

However, previous studies have shown that some dermaseptins and their derivatives are active against other viral strains. For instance, DRS-S1, along with its derivatives, exhibits a potential activity to eliminate pathogens associated with genital infections, such as the human papillomavirus (HPV) and the herpes simplex virus (HSV) [56]. Furthermore, derivatives of DRS-S4 have demonstrated antiviral activity against both Herpes Simplex Virus 1 and 2 [51,54]. Both dermaseptins DRS-S4 and S9, exhibit *in vitro* activity against the HIV-1 virus. They act by inhibiting the attachment of HIV to endometrial cells, its uptake by dendritic cells and preventing its transmission to T lymphocytes [53]. Lorin et al. suggest that DRS-S4 may play a role in the initial phase of viral replication, as they observed a smaller reduction in HIV levels once T cells were infected. The substitution of methionine by lysine at position 4 of DRS-S4 in order to reduce cytotoxicity for mammalian cells did not alter the anti-HIV activity observed [53]. Several recent studies have reported that many antiviral peptides can interact directly with viral particles, resulting in inactivation of virions. This represents a major antiviral mechanism for these peptides [57,58]. In general, the composition and electrical potential of the membrane constitute two crucial factors governing the affinity of this cationic peptide towards viral membranes [18,27].

The mechanism of action of dermaseptins against enveloped viruses can mainly be concluded as induction of viral envelope damage and inhibition of early virus attachment to host cells. To demonstrate that the viral membrane becomes permeable in the presence of active peptides, a sensitivity test of viral RNA (RT-PCR) to RNAse and/or propidium monoazide could be conducted on samples previously incubated with these antiviral peptides. However, previous research has provided a better understanding of the interaction of AMPs with their antiviral target. However, cathelicidins (LL-37) and BR2GK (Brevinin-2GHk) have been tested for their anti-ZIKV effects. He Miao et al. demonstrated the antiviral efficacy of cathelicidins (LL-37) and their derivatives against ZIKV, with significantly lower cytotoxicity observed in the derivatives compared to the native molecule. This effect was achieved by reducing infectious viral particles and inactivating the interferon IFN pathway [59]. Another study conducted by Xiong et al. reported that BR2GK (Brevinin-2GHk) directly inactivated ZIKV by disrupting the envelope integrity and can also penetrate the host cell membrane to inhibit the intermediate stage of ZIKV infection [60].

Table 3
Selectivity index of different dermaseptins for HMC3 cells.

Peptides	CC_{50}	IC_{50}	IS
$K_4K_{20}K_{27}S4$	33.60	0,82	40.97
$K_4K_{20}S4$	20.89	0,42	49.73
B2	ND	ND	ND
K_3K_4B2	ND	ND	ND
K4S4 (1–16)	37.30	21.71	1.71

CC_{50}: Concentration of the peptide (μg/ml) which causes 50 % cytotoxicity of HMC3 cells; **IC_{50}**: Concentration of the peptide (μg/ml) which induces 50 % reduction of HMC3 cells infected by ZIKV PF13; **IS**: Selectivity index: CC_{50}/IC_{50} ratio; **ND**: Not Determined.

4. Conclusion

In conclusion, the present findings show that our dermaseptines from the family of S4 have significant and selective antiviral effects against ZIKV PF13. This study also revealed that the cytotoxicity of these modified peptides was concentration dependent. We can conclude that DRS-S4 are active at low doses of 3 μg/ml against Zika virus. They inactivate the virus before infection of HMC3 cells, thus suggesting a mode of interaction of the peptide with the viral envelope. Collectively, these small molecules may have potential for use as safe antiviral agents.

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CRediT authorship contribution statement

Houda Haddad: Writing – original draft, Visualization, Software, Methodology, Investigation, Conceptualization. **Frédéric Tangy:** Validation, Supervision, Methodology, Investigation, Conceptualization. **Ines Ouahchi:** Writing – review & editing, Validation. **Wissal Sahtout:** Investigation, Formal analysis, Data curation, Conceptualization. **Bouraoui Ouni:** Visualization, Investigation, Formal analysis, Conceptualization. **Amira Zaïri:** Writing – review & editing, Validation, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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