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ORIGINAL ARTICLE

Design of antimicrobial peptides from a cuttlefish database

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Abstract

No antimicrobial peptide has been identified in cephalopods to date. Annotation of transcriptomes or genomes using basic local alignment Search Tool failed to yield any from sequence identities. Therefore, we searched for antimicrobial sequences in the cuttlefish (*Sepia officinalis*) database by in silico analysis of a transcriptomic database. Using an original approach based on the analysis of cysteine-free antimicrobial peptides selected from our Antimicrobial Peptide Database (APD3), the online prediction tool of the Collection of Anti-Microbial Peptides ($CAMP_{R3}$), and a homemade software program, we identified potential antibacterial sequences. Nine peptides less than 25 amino acids long were synthesized. The hydrophobic content of all nine of them ranged from 30 to 70%, and they could form alpha-helices. Three peptides possessed similarities with piscidins, one with BMAP-27, and five were totally new. Their antibacterial activity was evaluated on eight bacteria including the aquatic pathogens *Vibrio alginolyticus*, *Aeromonas salmonicida*, or human pathogens such as *Salmonella typhimurium*, *Listeria monocytogenes*, or *Staphylococcus aureus*. Despite the prediction of an antimicrobial potential for eight of the peptides, only two—GR_{21} and KT_{19}—inhibited more than one bacterial strain with minimal inhibitory concentrations below 25 µM. Some sequences like VA_{20} and FK_{19} were hemolytic, while GR_{21} induced less than 10% of hemolysis on human blood cells at a concentration of 200 µM. GR_{21} was the only peptide derived from a precursor with a signal peptide, suggesting a real role in cuttlefish immune defense.

Keywords Antimicrobial peptides · Design · *Sepia officinalis* · Transcriptome · Predictive tools

Introduction

Gramicidin was the first ever discovered antimicrobial peptide (AMP). That was back in 1940, when it was isolated from *Bacillus brevis* (Dubos and Cattaneo 1939; Hotchkiss and Dubos 1940). Since then, the search for AMPs has kept on, year after year. AMPs are key components of the immune system of plants and animals (Zasloff 2002; Boman 2003; Hancock and Sahl 2006). These peptides act rapidly against a large range of targets including bacteria, fungi, viruses, and parasites; some of them are more efficient than antibiotics (Yeaman 2003; Boman 2003). There is a large diversity of AMPs. Although the smallest AMPs are the anionic dipeptide Gageotetrin A from a marine bacterium (*Bacillus subtilis*) (Tareq et al. 2014) and Peptide F3 isolated from kefir (Miao et al. 2016), most of them are cationic and between 10 and 40 amino acids long. There are three main groups: (1) linear peptides forming an amphipathic α-helix and deprived of cysteine residues, e.g. cecropins (Steiner 1982), magainin-2 (Bechinger et al. 1993), or piscidins (Campagna et al. 2007; Houyvet et al. 2018), (2) cyclic

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peptides containing β-sheets, e.g. hepcidin (Lauth et al. 2005), or combined α-helices and β-sheets, e.g. drosomycin (Landon et al. 1997) or human β-defensin 2 (Harder et al. 1997; Hoover et al. 2000), and (3) peptides with an over-representation in proline and/or glycine residues, e.g. the proline-rich peptide PR39 (Agerberth et al. 1991).

In living organisms, AMPs are expressed from AMP genes constitutively or in response to infection (Hiemstra and Zaat 2013). AMPs could also be derived from the cleavage of bulky proteins like buforins extracted from histone H2A (Cho et al. 2009) or astacidin, the C-terminal end of crayfish hemocyanin (Lee et al. 2003).

AMPs are well described in Mollusks, particularly in bivalves (Destoumieux-Garzón et al. 2016; Zannella et al. 2017). Several AMPs rich in cysteine residues have been identified in mussels, e.g. mytilins, myticins, defensins, mytimacins (Mitta et al. 2000; Gerdol et al. 2012). In oyster, different AMPs have been described including defensins, big defensins, proline-rich peptides, and molluscidin (Bachère et al. 2004). In gastropods, defensin-like peptides have been identified in *Haliotis discus discus* (De Zoysa et al. 2010) or *Achatina fulica* (Zhong et al. 2013), and proline-rich peptides have been isolated from the hemolymph of *Rapana venosa* (Dolashka et al. 2011). In Gastropods and Cephalopods, a few proteins, e.g. Egg Case Proteins Sep-ECPs (Cornet et al. 2015b) or hemocyanin (Kremer et al. 2014; Dolashka et al. 2016) have demonstrated antibacterial activity. Hemocyanin is also at the origin of antimicrobial peptides, as demonstrated in abalone (*Haliotis* sp.) (Zhuang et al. 2015).

Although AMPs are produced by living organisms, recent research has focused on synthetic peptides, which can be derived from natural peptides to improve their activity, stability, and/or selectivity (Chen et al. 1988; Carmona et al. 2013; Mura et al. 2016). Some of them are de novo peptides like K4 (Duval et al. 2009). In parallel with the significant and consistent increase of the number of available antimicrobial peptides, online databases have been set up. Among these databases, specific ones are targeted to one AMP family, e.g. defensins (Seebah et al. 2007) (http://defensins.bii.a-star.edu.sg), or to AMPs from a given kingdom, e.g. bactibase (Hammami et al. 2010) (http://bactibase.pfba-lab-tun.org/main.php). Generalist databases have also been developed. They associate statistical tools or prediction tools such as the "Antimicrobial Peptide Database" APD (http://aps.unmc.edu/AP/main.php) (Wang and Wang 2004), recently updated (Wang et al. 2016), and the "Collection of Anti-Microbial Peptides" CAMP (http://www.camp.bicnirrh.res.in/) (Thomas et al. 2010). InverPep, a database on invertebrate antimicrobial peptides, has recently been developed: (http://ciencias.medellin.unal.edu.co/gruposdeinvestigacion/prospeccionydisenobiomoleculas/InverPep/public/home_en) (Gómez et al. 2017). Around fifteen other active databases also facilitate AMP analysis. They represent a considerable resource that we utilize to identify new AMPs from an atypical animal in which no AMP has ever been discovered.

In Cephalopods, especially in cuttlefish (*Sepia officinalis*), there is a knowledge gap about immune components (Castillo et al. 2015). However, the study performed by Cornet and collaborators (2015a) identified several signaling components from the NF-κB pathway. Besides, no humoral effector has been identified yet in this clade. BLAST annotation of available transcriptomes or of the *Octopus bimaculoides* genome did not yield any AMPs from sequence identities, even for AMPs such as defensins whose sequences associating cysteines are relatively well conserved in Mollusks (De Zoysa et al. 2010).

In this context, we developed an in silico approach to identify new AMP candidates from the cuttlefish transcriptome and to overcome the difficulties related to the classical method using animals. This article describes the methodological approach we applied, based on the analysis of cuttlefish transcriptome including more than 61,900 transcripts involved in secretion of neurotoxic saliva (Cornet et al. 2014), egg case formation (Cornet et al. 2015c), the immune system associated to accessory nidamental glands (Cornet et al. 2015a), and the neuro-endocrine system (Zatylny-Gaudin et al. 2016). We specifically searched for linear AMPs forming a potential amphipathic α-helix and deprived of cysteine residues to facilitate their synthesis. To target these peptides in cuttlefish, we analyzed sequences of cysteine-free AMPs available in databases to determine search criteria, and used predictive tools.

Materials and methods

Peptide design

The methodology used in this study is illustrated by the pipeline in Fig. 1. For the first step, we used APD (Wang et al. 2016), which mainly includes natural peptides, to determine selection criteria. We analysed 811 cysteine-free AMPs (Online resource 1), 11–25 residues long, potentially able to form an alpha-helix structure or no such structure at all; these features were compatible with chemical synthesis. From these 811 AMPs including AMPs identified in Mollusks, Arthropods, Tunicates, Fish, Amphibians, Reptiles and Mammals, we used the occurrence of certain amino acids or certain associations of amino acids as a search criterion through the cuttlefish database (Sequences are available from Genbank under Bioproject PRJNA242869 and Biosample accession No: SAMN02709769) in PepTraq. PepTraq is a homemade software program developed to perform in silico analyses of large batches of genomic, transcriptomic, or proteomic

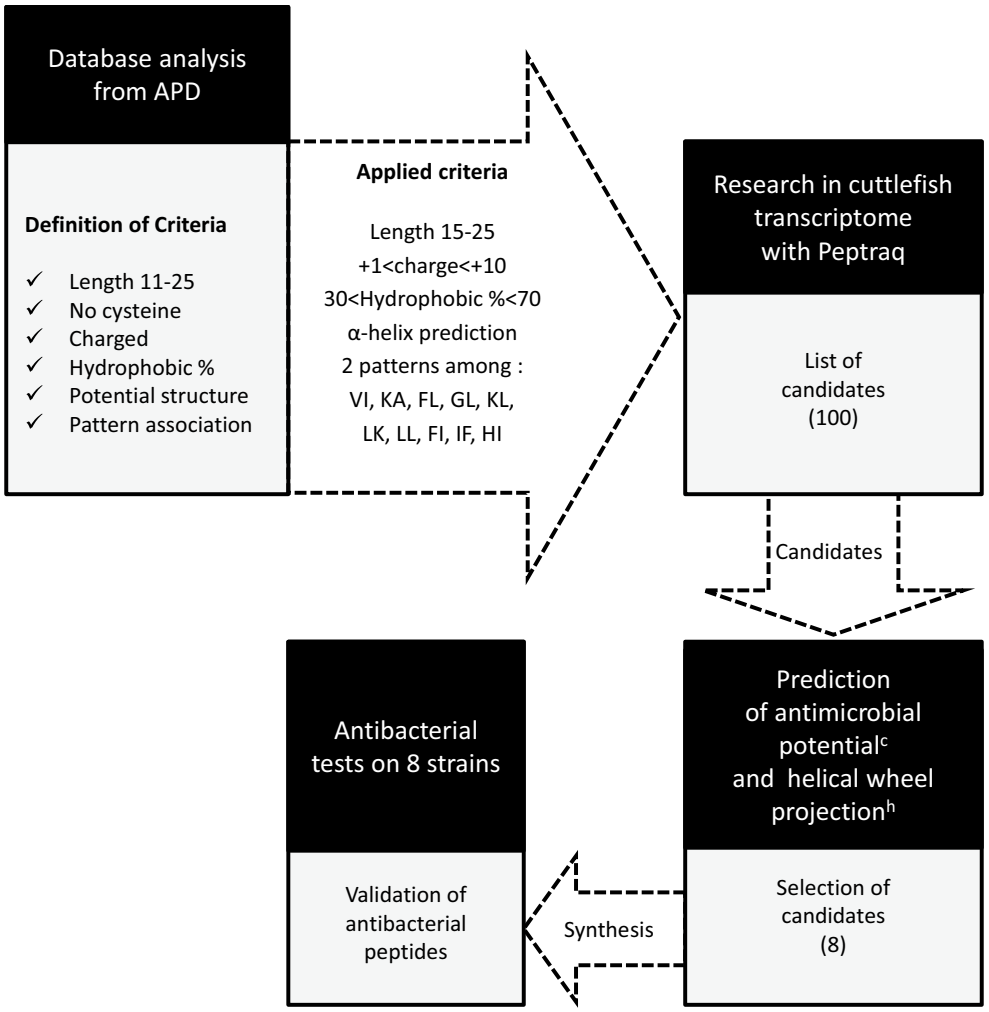


Fig. 1 Pipeline of the global methodology used to select potential antibacterial peptides in the cuttlefish transcriptome. [c]CAMP (http://www.camp.bicnirrh.res.in/), [h]Heliquest (http://heliquest.ipmc.cnrs.fr/), APD (http://aps.unmc.edu/AP/main.php)

data based on structural criteria (peptide size, occurrence of certain amino acids, number or percentage of certain amino acids, occurrence of consensus sequences, net electrical charge of precursors or peptides). It previously allowed us to identify the cuttlefish neuropeptidome (Zatylny-Gaudin et al. 2016). We evaluated the antibacterial potential of the selected cuttlefish sequences using available tools in the CAMP database website (Hanif Waghu et al. 2016) (http://www.camp.bicnirrh.res.in/prediction.php). The following four algorithms were selected: Support Vector Machine (SVM), Random Forests (RF), Artificial Neural Network (ANN), and Discriminant Analysis (DA). To complete the sequence analysis, we determined the net charge, total hydrophobic ratio and molecular weight of each predictive structure using APD (Wang et al. 2016). The helical wheel projection diagrams were predicted using Heliquest (Gautier et al. 2008) (http://heliquest.ipmc.cnrs.fr/cgibin/ComputParamsV2.py).

Peptide synthesis

Nine selected peptides (eight potential antimicrobial peptides and one peptide without potential antimicrobial activity) were synthesized by GENECUST (Dudelange, Luxembourg) with > 97% overall purity.

Antimicrobial assays

The minimal growth inhibitory concentrations (MICs) of the peptides were evaluated on several bacteria supplied by the "Collection de l'Institut Pasteur" (CIP), namely *Listeria monocytogenes* (CIP 110871), *Enterococcus faecalis* (CIP 76.117), *Staphylococcus aureus* (CIP 53.1 56), *Bacillus megaterium* (CIP 51.17), *Escherichia coli* (CIP 54.8T), *Salmonella typhimurium* (CIP 103446), *Vibrio alginolyticus* (CIP 109819), and *Aeromonas salmonicida* (CIP 103209T). Bacteria were cultured in adapted media: Brain

Heart Infusion (BD) 37 g/L, pH 7.4 for *L. monocytogenes*; Luria–Bertani (peptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L) for *E. faecalis*, *B. megaterium*, and *E. coli*; Columbia broth (Conda) 35 g/L, pH 7.4 for *S. aureus*; Trypticasein Soy Broth or TSB (Conda) 40 g/L, pH 7.3 for *S. typhimurium* and *A. salmonicida,* and Marine Broth (Conda) 40.2 g/L, pH 7.6 for *V. alginolyticus*. MICs were determined in triplicate by liquid growth inhibition assays (Hetru and Bulet 1997). Briefly, 10 µL of peptide solution were incubated in microtiter plates with 100 µL of a bacterial suspension at a starting optical density at 600 nm (OD_{600}) of 0.001 in Columbia broth in the same culture medium as before for *S. aureus*, i.e. Columbia broth (Conda) 35 g/L, in TSB for *S. typhimurium*, and *A. salmonicida*, in Poor Broth (peptone 10 g/L, NaCI 5 g/L, pH 7.4) for *E. faecalis*, *B. megaterium*, and *E. coli*, in Saline Poor Broth (peptone 10 g/L, NaCI 15 g/L, pH 7.2) for *V. alginolyticus,* and in Poor Brain Heart Infusion (BD) 18.5 g/L (peptone 5 g/L, NaCl 2.5 g/L, pH 7.4) for *L. monocytogenes*. Bacterial growth was evaluated by measuring optical density at 595 nm after 16 h incubation at 30 °C (20 °C for marine bacteria). The different peptide concentrations were tested at 10 µM, 50 µM, 100 µM, and 250 µM. MICs were expressed in µM as an [a]–[b] concentration interval, where [a] was the last concentration with microbial growth and [b] was the first concentration with 100% growth inhibition. MBCs (minimal bactericidal concentrations) were expressed in µM as a [a]–[b] concentration interval, where [a] was the last concentration with microbial growth and [b] was the first concentration that killed 100% of the bacteria.

Hemolytic activity

Hemolytic activity was determined as described by (Duval et al. 2009) in triplicate. Briefly, human red blood cells (hRBCs) were washed twice in PBS, and then diluted to obtain a solution of 1% of erythrocytes. 90 µL of the solution were incubated with 90 µL of different peptide concentrations ranging from 10 µM to 250 µM, dissolved in PBS buffer for 1 h at 37 °C. Finally, hemolysis was determined by measuring the optical density of the supernatant at 415 nm. Zero hemolysis (blank) and 100% hemolysis were determined in PBS buffer and 1% Triton X-100, respectively.

Circular dichroism

The CD spectra of GR_{21} (0.1 mg/mL) were measured in potassium phosphate buffer in the presence or in the absence of trifluoroethanol 30 or 75% (v/v) on a JASCO J810 CD spectrophotometer in a 1-mm path length cell. CD spectra were recorded from 190 to 250 nm. CD spectrum analyses and relative helix contents were deduced using the online BESTSEL software program developed by Micsonai and collaborators (2015).

Results and discussion

The 811 AMPs extracted from the APD included frog AMPs like magainins (Zasloff 1987), temporins (Simmaco et al. 1996), aureins (Rozek et al. 2000), and maximins (Lee et al. 2005), venomous arthropod AMPs like eumenitin (Konno et al. 2006), mastoporans (Čeřovský et al. 2008), snail AMPs (Dolashka et al. 2011), styelins tunicate AMPs (Lee et al. 1997), fish AMPs like piscidins (Silphaduang and Noga 2001; Lauth et al. 2002), or histone-derived peptides (Robinette et al. 1998), and also a few synthetic peptides like CM15, a hybrid of cecropin and melittin (Andreu et al. 1992), or de novo peptides like K4 (Duval et al. 2009). All these peptides represented more than 27% of the APD and 65% of the peptides between 11 and 25 residues long; all AMPs of this length, whether containing cysteine(s) or not, corresponded to 40% of the APD peptides. The amino acids mostly present in the 811 sequences were glycine and hydrophobic residues, particularly leucine, isoleucine, alanine and phenylalanine. The majority of peptides were cationic, and lysine was the most frequently charged residue. These physic-chemical properties including hydrophobicity of amino acid residues, as well as the charge largely influence antibacterial activity (Zelezetsky and Tossi 2006; Grau-Campistany et al. 2015). Very often, i.e. in more than 10% of the 811 sequences, these amino acids associated with hydrophobicity or positive charge made up recurring patterns like KA, GL, KL, LK, LL, or VI.

We selected sequences from the PepTraq cuttlefish database, using four main structural criteria: (1) a size ranging from 11 to 25 residues; (2) a positive charge; (3) richness in leucine, isoleucine, alanine, glycine or lysine; and (4) the presence of two recurrent patterns among KA, GL, KL, LK, LL, VI or FI, IF, HI and IH; i.e. common patterns to piscidins and temporins. We selected 100 sequences combining these four criteria from the cuttlefish transcriptome, and evaluated their antimicrobial potential with CAMP algorithms. Eight sequences were validated (Table 1), and a negative sequence (FD_{20}) was added as a control.

Sequences were between 16 and 21 amino acid residues long. FI_{16} was the shortest peptide, and GR_{21} the longest. Molecular weights ranged from 1872.23 (AT_{20}) to 2651.11 (GR_{21}). Total hydrophobic ratios ranged between 33 and 68%, and total net charges from +2 (KG_{17}) to +7 (GR_{21}). We evaluated the antimicrobial potential of the eight cationic peptides FI_{16}, KG_{17}, FK_{19}, KT_{19}, AT_{20}, GT_{20}, VA_{20} and GR_{21} with the four CAMP algorithms (Table 1).

The anionic peptide FD_{20} was synthesized as a non-antimicrobial control. All sequences were able to form alpha

Table 1 Peptides selected from the cuttlefish database

Name	Length (AA)	Sequence	MM[a]	HR[a]	Prediction of α-helix and NBH[a]	Charge[a]	Prediction for antimicrobial peptide[b]			
							SVM	RFC	ANN	DAC
FI_{16}	16	FIFHIIRFFNFRVFRI	2172.65	68%	Yes-8	3	0.725	0.77	AMP	0.980
KG_{17}	17	KIFDAEILLNGKRKGLG	1872.23	41%	Yes-5	2	0.795	0.606	AMP	0.794
FK_{19}	19	FIKIFHHIFKGNPKFSIIK	2314.83	47%	Yes-6	4	0.969	0.85	AMP	0.996
KT_{19}	19	KIRFTRTVSRLLKAALAST	2132.57	47%	Yes-7	5	0.768	0.787	AMP	0.964
AT_{20}	20	AKAFKKAFEKLAAVVPFGGT	2080.48	55%	Yes-5	3	0.817	0.749	AMP	0.974
GT_{20}	20	GKSNKPALTLIQARILKHKT	2217.67	35%	Yes-6	5	0.945	0.6805	AMP	0.939
VA_{20}	20	VILTRFRFLNRIVEPLLKKA	2427.02	55%	Yes-8	4	0.823	0.8025	AMP	0.994
GR_{21}	21	GSTSFHLIYNKWFAVKRRRKR	2651.11	33%	Yes-4	7	0.932	0.792	AMP	0.961
FD_{20}	20	FFHHIFDPQIKSGLLVSMYD	2394.78	45%	Yes-7	-1	0.081	0.024	NAMP	0.124

MM molecular mass, *HR* hydrophobic ratio, *NBH* number of residues in the same hydrophobic surface, *SVM* Support vector machine classifier, *RFC* random forest classifier, *ANN* artificial neural network, *DAC* discriminant analysis classifier

[a]Parameters and prediction from APD

[b]Prediction evaluated form algorithms of CAMP, AMP anti-microbial peptide, NAMP no anti-microbial peptide

helices and possessed between 4 and 8 residues on the same hydrophobic surface according to the APD prediction (Table 1). We used Heliquest to calculate helix properties. Figure 2 shows the helical wheel projection of the cationic peptides. In this conformation, some of the peptides revealed a hydrophobic face that differed from the APD prediction. Only AT_{20}, VA_{20}, FK_{19} and KT_{19} seemed to adopt an amphiphilic α-helix.

A BLASTP (Basic Local Alignment Search Tool for Proteins) from CAMP website revealed similarities between three sequences and piscidins. Peptide FI_{16} displayed 43.8% identity (81.2% similarity) with epinecidin-1 of *Epinephelus coioides* (Lin et al. 2009), and FK_{19} and FD_{20}, respectively, displayed 47.4% identity (57.9% similarity) and 23.1% identity (34.6% similarity) with moronecidin of *Siniperca chuatsi* (Sun et al. 2007) (Fig. 3). The acquisition of these three peptides met our PepTraq criteria (FI, IF, HI and IH) and validated our methodology. Another sequence revealed local alignment of AT_{20} [37.0% identity (55.6% similarity)] with BMAP-27 derived from cathelicidin of *Bos Taurus* (Skerlavaj et al. 1996) (Fig. 3). The other sequences KG_{17}, KT_{19}, VA_{20}, GT_{20} and GR_{21} shared no homology with known peptides.

The antibacterial assays were performed on 8 bacteria including both Gram-positive and Gram-negative bacteria. Some were human pathogens like *Listeria monocytogenes* (Marquis et al. 2015) and *Staphylococcus aureus* (Lowy 1998), while others such as *Vibrio alginolyticus* or *Aeromonas salmonicida* are pathogenic for aquatic organisms (Sangster and Smolowitz 2003; Menanteau-Ledouble et al. 2016). Out of the nine peptides, five inhibited the growth of at least one bacterial strain, often at high concentrations (Table 2). This was the case for FK_{19} and the anionic FD_{20} which inhibited *Escherichia coli* up to 150 µM and *Enterococcus faecalis* between 100 and 150 µM, respectively. Neither was bactericidal. Besides, VA_{20} inhibited the two bacteria *Bacillus megaterium* and *E. coli* at 100 µM and 25–50 µM, respectively. It was otherwise bactericidal on the two strains at 100 µM. Finally, KT_{19} and GR_{21} were the most active antibacterial peptides: they inhibited all bacteria, except *Vibrio alginolyticus* that was not sensitive to GR_{21}. Both peptides showed low MICs and MBCs under 25 µM on several strains including *A. salmonicida, E. coli, B. megaterium*, and *S. aureus.* They both had a broad antibacterial activity spectrum, while the spectrum of other active peptides was narrower. Although Strandberg and collaborators (2015) demonstrated the influence of hydrophobic residues on the antibacterial activity of magainin 2, the most hydrophobic sequence FI_{16}, as well as AT_{20} did not appear to have any antibacterial activity despite their similarities with antimicrobial peptides like epinecidin-1 or BMAP 27.

We evaluated hemolytic activity against human red blood cells. Results are reported in Fig. 4. No hemolytic activity was induced by any of the peptides at a low concentration of 5 µM, but hemolysis occurred when concentrations increased. At 50 µM, GR_{21} and FI_{16} induced less than 5% of hemolysis, while VA_{20} and FK_{19} induced 10 and 43% hemolysis, respectively. At 100 µM, KT_{19} induced slight hemolysis (< 10%), and 21% at 200 µM. Finally, the most hemolytic peptide was FK_{19}: it caused more than 50% of hemolysis from 100 µM. FK_{19} has a conserved pattern (FHHIFXG) in common with moronecidins, in particular with moronecidin of *Morone saxatilis,* which are hemolytic on sheep and human red blood cells (Lauth et al. 2002). This pattern, therefore, appears to be involved in interactions with eukaryotic membranes.

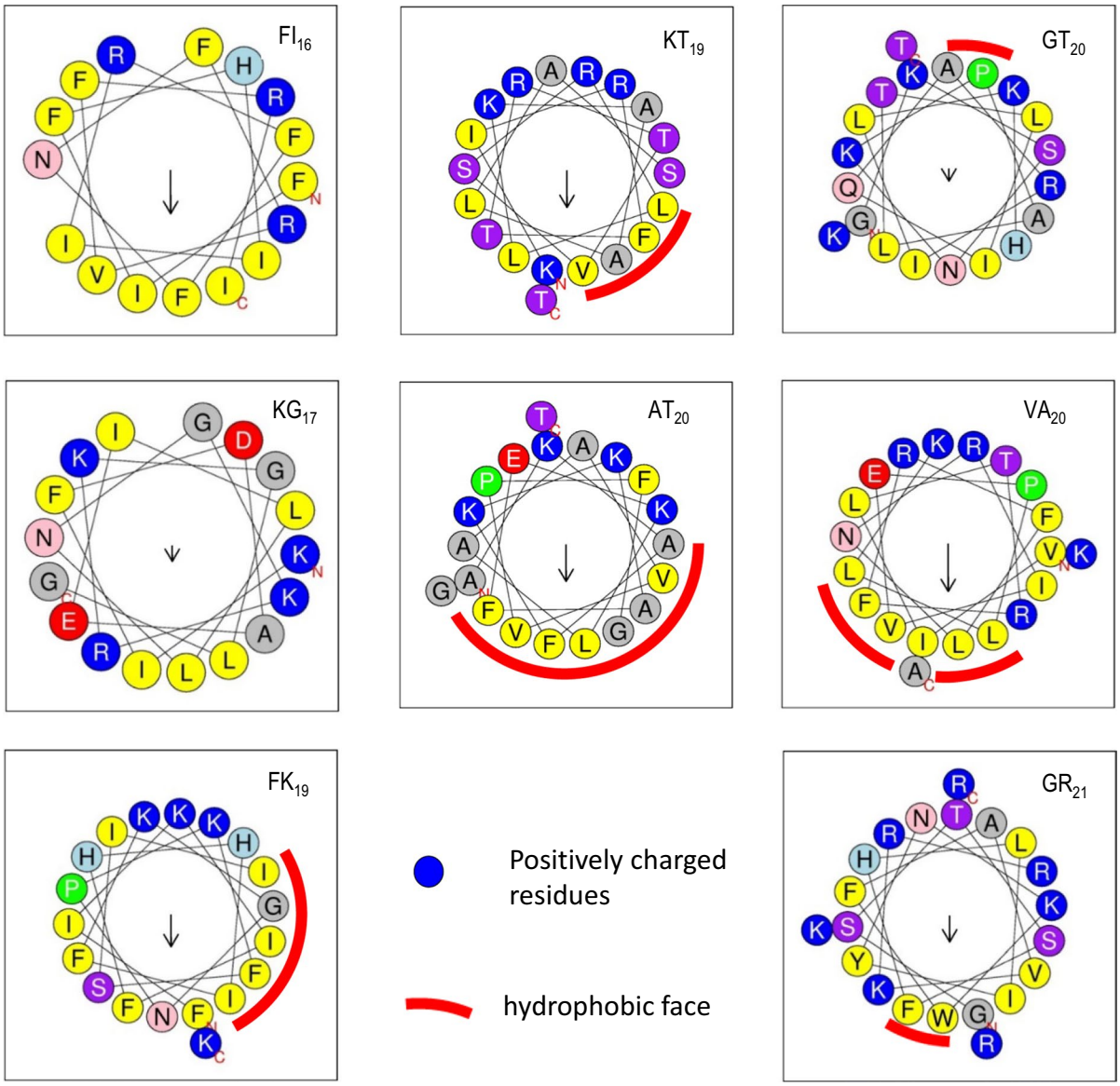


Fig. 2 Helical wheel diagram of FI_{16}, KG_{17}, FK_{19}, KT_{19}, AT_{20}, GT_{20}, VA_{20} and GR_{21} peptides. The helical wheel projections were performed using the HeliQuest online program: http://heliquest.ipmc.cnrs.fr. Yellow, hydrophobic amino acids; blue, basic residues; green; special residues; red, acidic residues; purple and pink, polar amino acids, arrow, hydrophobic moment

FI_{16}	**FIFHII**RFFNFRVFRI
Ec-epinecidin-1	**FIFHII**KGLFHAGKMIHGLVTRRRH
FK_{19}	FIK**IFHHIFK**GNPKFSIIK
Sc-moronecidin	**IFHHIFK**GIVHVGKT**I**HRLVTG
FD_{20}	F**FHHI**FFFHHIFDPQ**I**KSGLLVSMYD
AT_{20}	GRFKRFR**K**K**FKK**L**F**K**KL**SP**V**I**P**LLHLG
Bt-BMAP-27	A**K**A**FKK**A**F**E**KL**AA**V**V**P**FGGT

Fig. 3 Alignment of the selected sequences. FI_{16} with Epinecidin-1 of *Epinephelus coioides* (Q6JWQ9), FK_{19} and FD_{20} and moronecidin of *Siniperca chuatsi* (Q2VWH5), and AT_{20} with the antimicrobial peptide BMAP-27 derived from cathelicidin of *Bos taurus* (P54228)

Besides, KT_{19} and GR_{21} were the antibacterial peptides with the largest activity spectrum both on Gram-negative and Gram-positive bacteria. The CD analysis confirmed that these two peptides could form a helical structure in the presence of trifluoroethanol (Fig. 5). They were also the most cationic peptides. GR_{21} was strongly active while it was the least hydrophobic peptide and the most cationic one, supporting the important role of the electrical charge for antibacterial activity. Most AMPs are usually cationic (Hancock and Lehrer 1998; Hancock and Diamond 2000), and many of them are involved in host defense (Brown and Hancock 2006).

Table 2 Antibacterial activity of the synthetic peptides

Peptide	Gram-positive								Gram-negative							
	Listeria monocytogenes		*Bacillus megaterium*		*Enterococcus faecalis*		*Staphylococcus aureus*		*Escherichia coli*		*Salmonella typhimurium*		*Vibrio alginolyticus*		*Aeromonas salmonicida*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
FI_{16}			–	–	–	–	–	–	–	–	–	–	–	–		
KG_{17}	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
FK_{19}	–	–	–	–	–	–	–	–	≥150	NB	–	–	–	–	–	–
KT_{19}	≥50	≥50	1–10	1–10	100–150	NB	10–25	25–50	1–10	1–10	75–100	75–100	25–50	25–50	10–25	10–25
AT_{20}	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
FD_{20}	–	–	–	–	100–150	NB	–	–	–	–	–	–	–	–	–	–
GT_{20}	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
VA_{20}	–	–	≥100	≥100	–	–	–	–	25–50	≥100	–	–	–	–	–	–
GR_{21}	25–50	25–50	1–10	1–10	75–100	100–150	5,10	10–25	1–10	1–10	≥50	≥50	–	–	≤5	≥50

MIC minimal inhibition concentration, *MBC* minimal bactericidal concentration, - no activity, *NB* not bactericidal

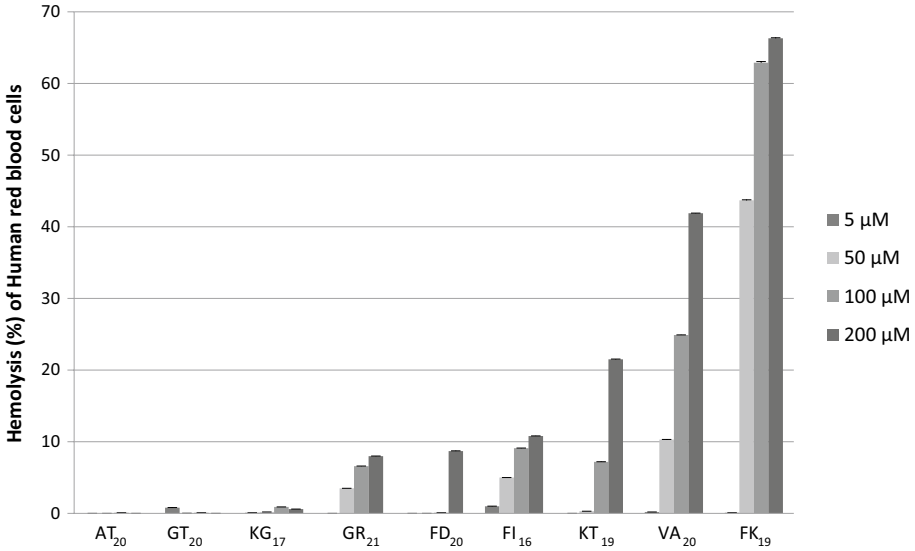
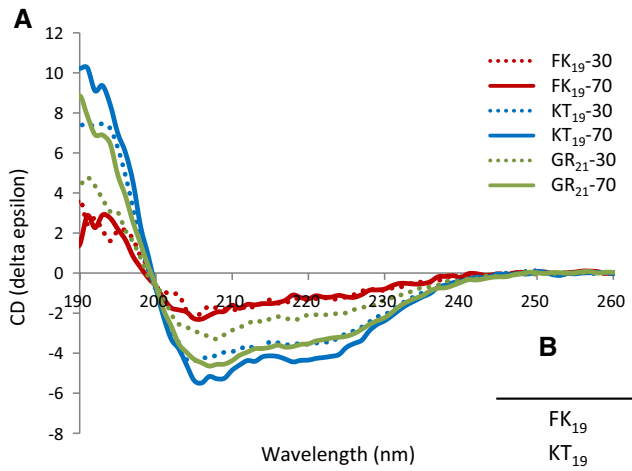


Fig. 4 Hemolytic assay. The hemolytic activity of all nine peptides was evaluated using human red blood cells (hRBCs)



B

	% of α-helix content	
	30% TFE	70% TFE
FK_{19}	9,80%	10,10%
KT_{19}	23,90%	31%
GR_{21}	20%	26%

Fig. 5 Circular dichroism analysis. **a** Spectra of GR21, FK19, and KT19 in the presence of trifluoroethanol 30 or 70%. **b** Calculated helix contents based on the online BESTSEL software program developed by Micsonai et al. (2015)

Although this work is not entirely conclusive for the discovery of cuttlefish AMPs, it opens up new research perspectives on AMPs. Moreover, the results obtained with GR21, which is the only peptide selected from a precursor with a signal peptide, suggest a physiological role of this peptide in cuttlefish immune defense. By targeting organs involved in immunity and peptides derived from a precursor with a signal peptide, this approach could be much more promising.

The strategy developed in this study allowed us to design antimicrobial peptides from a cuttlefish database. Only four of the eight designed peptides had in vitro antibacterial properties despite the potential antimicrobial activity predicted by all four CAMP algorithms. Predicting the antibacterial potential of peptides remains difficult, taking into account the hydrophobicity and the net charge which condition antibacterial activity. Studies about the use of predictive tools such as CAMP are scarce. Sharma and collaborators (2015) carried out an in-depth study to compare two antibacterial predictive tools, CAMP versus DBAASP (Database of Antimicrobial Activity and Structure of Peptides: https://dbaasp.org/home) to target *Acinetobacter baumannii* (Sharma et al. 2015). Nevertheless, their study focused on peptides already described to be antibacterial. In the present study, we tried to design new AMPs from a database that did not contain known AMPs. Our results also show that chosen predictive tools are not optimal. For peptides like FI_{16}, KG_{17}, GT_{20} and AT_{20} predicted to be antibacterial, in vitro tests on eight bacteria failed to validate this potential. Nevertheless, they might act against other targets like fungi or protozoa. On the contrary, FD_{20}, though not strongly active, inhibited the growth of one bacterium while it had not been predicted to have antibacterial activity. The same was observed for peptide histatin-8: although it is a human antibacterial peptide (Vila-Farres et al. 2012), it was not predicted to be antibacterial (Sharma et al. 2015). The use of AMPA, an automated web server for predicting protein antimicrobial regions, could have improved our selection criteria. The online tool http://tcoffee.crg.cat/apps/ampa used after our study showed that the three KT_{19}, FK_{19}, and GR_{21} peptides had potential antimicrobial regions. It is, therefore, important to use multiple predictive tools to optimize the search for new antimicrobial compounds.

Otherwise, the present study shows how complex it is to decipher what is exactly needed for a peptide to be antibacterial. Using tools such as Heliquest is interesting to visualize the spatial arrangement of the different peptides. The common feature between our most active peptides is the clear segregation of the hydrophobic moiety from the cationic core as compared to the other peptides. Balancing peptide hydrophobicity and charge distribution is probably a determining factor of in vitro activity of AMPs.

To conclude, the methodology developed in this study allowed us to design two new antibacterial peptides that have a large spectrum of antibacterial activity but do not induce hemolysis. Furthermore, their MICs and MBCs are low for some strains, which render them more selective for these sensitive strains. It would be interesting to further study these two peptides to investigate their mode of action. As regard to peptide GR_{21}, which comes from a precursor with a signal peptide upstream of the antibacterial sequence, it would be interesting to study its biological function in cuttlefish to confirm its involvement in immunity. This approach also represents an alternative to classic methods such as purification from peptide extracts which needs animal material and is labor-intensive.

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Compliance with ethical standards

Conflict of interest The authors declare no competing financial interest.

Informed consent The manuscript was written through contributions of all authors. All authors have given approval of the final version of the manuscript.

Ethical statement The article does not contain any studies in patients by any of the authors. This article contains studies with human or animal subjects that are all approved of by the Ethics Committee.

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