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Characterization of a possible uptake mechanism of selective antibacterial peptides

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Selective antibacterial peptides containing less than 30 amino acid residues, cationic, with amphipathic properties, have been the subject of several studies due to their active participation and beneficial effects in strengthening the immune system of all living organisms. This manuscript reports the results of a comparison between the group of selective antibacterial peptides and another group called "cell penetrating peptides". An important number of the selective antibacterial peptides are cell penetrating peptides, suggesting that their toxicity is related to their uptake mechanism. The verification of this observation also includes the adaptation of a method previously published, called Polarity index, which reproduces and confirms the action of this new set of peptides. The efficiency of this method was verified based on four different databases, yielding a high score. The verification was based exclusively on the peptides already reported in the databases which have been experimentally verified.

Key words: Polarity index method; cell penetrating peptides; selective antibacterial peptides

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INTRODUCTION

Selective cationic amphipathic antibacterial peptides (SCAAP) (Del Rio *et al.*, 2001; Juretić *et al.*, 2009) feature very high toxicity towards bacteria and a minimal toxicity towards mammalian cells. Although so far the reason for this selective toxicity is not known, it makes them potentially important for the production of drugs for the pharmaceutical industry. SCAAP were initially isolated in living organisms and then tested experimentally in laboratory, but after a few decades the number of SCAAP was reduced exponentially; therefore, new bioinformatics tools had to be used to build them synthetically. This work uses the 30 SCAAP reported by Del Rio *et al.* (Del Rio *et al.*, 2001) that recently this research group was able to identify through a method called Polarity index (Polanco *et al.*, 2012; Polanco *et al.*, 2013), showing a high discriminative efficiency measuring a single physicochemical property, polarity (or electronegativity), which informs of the electromagnetic balance of the peptide.

Parallel to these works, several research groups have studied some peptides called Cell Penetrating Peptides (CPP) characterized by being short, between 10 and 30 amino acids in length, and penetrating the cell membrane without displaying any specificity (Gautam *et al.,* 2012), which makes them excellent candidates for Trojan peptides (Derossi *et al.*, 1998). Trojan peptides are a union of two or more peptides with different characteristics that together make a peptide with certain desirable features; they are usually used as internment peptides in pathogenic organisms.

In recent years, with the complete information provided by the CPPsite database (Gautam *et al.*, 2012), the study of CPP was encouraged making it possible to compare them with other peptide groups and increase the understanding of these fundamental functional units. This work derived from the compilation of this information, since it allowed us to study the relationship between CPP and SCAAP, finding that the CPP with non-endocytic pathway uptake mechanism exhibit a high correlation with SCAAP, particularly with the SCAAP set from Del Río *et al.* (Del Rio *et al.* 2001). This led us to define the group studied here as SCAAP-CPP, whose first property is to belong to both groups.

According to the definition of the SCAAP-CPP set, this work was mainly aimed to find the correlation in the sequences of both groups and to analyze the pattern found in their polarity matrix (Polanco *et al.*, 2013) with the Polarity index method (Polanco *et al.*, 2012). The method testing to identify SCAAP-CPP was exhaustive, as four databases were inspected: APD2 (Wang & Wang, 2009), CPPsite (Gautam *et al.*, 2012), AVPpred (Thakur *et al.,* 2012) and Uniprot (Magrane & Uniprot, 2011), finding that the efficiency of the SCAAP-CPP identification is remarkably high.

MATERIALS AND METHODS

Polarity index method description. Polarity index method is a supervised type mathematical algorithm (Jones & Sivaloganathan, 2011), that carries out an extensive analysis of the physico-chemical property polarity; although there are multiple algorithms evaluating this property on its own or in combination with other properties, this particular algorithm expresses polarity using a fourth order square matrix (16 items representing the four polar groups with 16 possible polar interactions P +, P–, N, NP), allowing observation of all possible polar interactions that can be extracted from the peptide linear sequence, reading it from left to right by pairs. For instance if we have the following sequence: HTWT

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Abbreviations: CPP, cell penetrating peptides; SCAAP, Selective cationic amphipathic antibacterial peptides

Table 1. Polarity matrix.

	P+	P–	N	NP
P+	1	3	6	4
P–	2	1	4	1
N	7	3	7	10
NP	3	1	10	7

Polarity matrix interaction in the polarity groups differentiated by their lateral chain to the sequence e.g. HTWTWTPICKSRSHEYKG RCIQDMDC-NAACVKESESYTGGFCNGRPPFKQCFCTKPCKRRAAATLRWPGW.

WTP ICKSR SHEY KGR CIQDM DC NAACV KESE SYTG GFC NGRP PFKQC FCTKP CKRR AAATL RW PGW, according to the numerical assignation by polar group, its equivalent would be: 134 34 3443 1313 1221 31 343 2423 3443 41232 323 334 333 1444 1334 331 431 121 444 3414 434 (Polanco *et al.*, 2012, Table 1). The matrix is created when reading the latter as indicated and the incidences are counted as shown in Table 2. This method differs from others by considering 16 metrics that make the information more extensive.

As the method has been already published (Polanco *et al.*, 2012), we will only point out the changes required to identify SCAAP-CPP (see Supplementary Material section). In this case the test plan was focused on two aspects: (a) to verify the SCAAP and CPP matches located in the databases and (b) to particularly identify the SCAAP-CPP using Polarity index method.

Polarity index method updates. Modifications to the source program. The Q[*i,j*] matrix in the source program (Polanco *et al.*, 2012) was substituted with Table 2, which is the entire peptide set of cell penetrating sequences with unique pathogenic action. Once Q[*i,j*] polarity matrix was finished, it was normalized to one. Under this rule we considered the cell penetrating peptides non-endocytic pathway type, taken from the CPPsite database (Gautam *et al.*, 2012). It is important to emphasize that we did not use the SCAAP group, or the union of SCAAP and CPP groups.

The rule in the source program (Polanco *et al.*, 2012) was substituted with the P[*i,j*] + Q[*i,j*] vector complying with the next rule: polar interaction 11 is present in the 7th position (Table 3).

APD2 database data preparation. From all 3636 peptides in APD2 database studied and classified we found the following with multiple action on: 149 Gram–ONLY, 1711 Gram+/Gram–ONLY, 315 Gram+ONLY, 141 cancer cells, 9 sperms, 88 HIV, 744 fungi, 21 insects, 244 mammalian cells, 47 parasites, 3 protists, 39 chemotaxis, 0 SCAAP, 125 virus; and 1059 peptides with single action on: 111 Gram–ONLY, 213 Gram+ONLY, 518 Gram+/Gram–ONLY, 20 cancer cells, 0 HIV, 88 fungi, 35 H1N1, 2 insects, 11 mammalian cells, 9 parasites, 1 protists, 0 chemotaxis, 30 SCAAP, 0 sperms and 21 virus. Multiple action peptide refers to action against several pathogens whereas single action peptide refers to action against one pathogen agent.

AVPpred database data preparation. 60 antiviral peptides were studied from the AVPpred database (Thakur *et al.,* 2012).

CPPsite database data preparation. 520 cell penetrating peptides were classified from CPPsite database (Gautam *et al.,* 2012) by their uptake mechanism as follows: 93 non-endocytic pathway, 22 endocytic pathway, and 405 of unknown pathway. Those peptides with different penetration mechanism included in CPPsite database were not considered.

SCAAP data preparation. The 30 SCAAP studied by Del Rio *et al.* were used (Del Rio *et al.*, 2001, Table 2 and Table 2A).

Uniprot database data preparation. 60 antiviral peptides were extracted from Uniprot Database (Magrane & Uniprot, 2011).

Table 2. Q[*i,j*] Polarity matrix

	P+	P-	N	NP
P+	0.1299774647	0.0045078886	0.0503380932	0.1502629668
P	0.0060105184	0.0000000000	0.0007513148	0.0075131478
N	0.0548459813	0.0030052592	0.0435762592	0.0555972941
NP	0.1359879822	0.0075131478	0.0653643906	0.2148760259

93 incidences of cell penetrating peptides of non-endocytic pathway type, taken from CPPsite database (*Gautam et al.,* 2012).

RESULTS

Polarity index method is an algorithm that determines the probable SCAAP-CPP non-endocytic pathway candidates, by using their polarity sequence. We applied the method to get the set of 30 SCAAP from the four databases mentioned, with the following results:

(i) Polarity index method excluded 2 peptides from the 30 SCAAP candidates from Del Rio *et al.* (Del Rio *et al.*, 2001) (Table 4, entries #9 and 21 with symbol ♦), these peptides have very different TI but the same sequence, it showed (11/13) 55% efficiency detecting SCAAP-CPP (Table 5).

(ii) It also excluded the 13 sub-classifications in APD2 (Table 5, columns: Gram+ONLY, Gram–ONLY, Gram+/Gram–, fungi, protists, parasites, insects, cancer cells, mammalian cells, virus, SCAAP, and chemotaxis, in blue), the classification in AVPprep (Table 5, in green), the sub-classification in Uniprot (Table 5, in red), and three sub-classifications in CPPsite (Table 5, in black).

(iii) Polarity index method also identified three SCAAP-CPP peptides (Table 4, entries #2, 4, and 5), whose experimental attributes are SCAAP and CPP simultaneously. Note that those SCAAP were not located in the CPP database.

DISCUSSION

The increasing number of pathogens resistant to multiple drugs that have been registered in the last 20 years (Rana, 2011), and the decrease in the number of effective antibacterial drugs, indicate that by the end of this decade there will be few antimicrobial drugs available for human population. There are no "magic bullets"; in fact, the possibility of losing the current antibacterial arsenal is more likely every day unless we reverse this trend. Bearing in mind that 67% of natural origin antibiotics (Donadio *et al.*, 2010) in clinical development are researched in small pharmaceutical companies with limited budgets, and as the major pharmaceutical companies focus their efforts on developing new antibacterial agents, we gather that only with a multidisciplinary strategy with novel bioinformatics algorithms acting as "first filter" in experimental tests, we will be able to face this real threat. The SCAAP-CPP is an antibacterial group with potential for the de-

Table 3. Polarity index method test.

Position	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
P[*i,j*] + Q[*i,j*] vector of study	(1,1)	(1,2)	(1,3)	(1,4)	(2,1)	(2,2)	(2,3)	(2,4)	(3,1)	(3,2)	(3,3)	(3,4)	(4,1)	(4,2)	(4,3)	(4,4)
Rule # 1 Polar interaction 11 is present at the 7th position							✓									

Polarity index method identification rule for SCAAP-CPP. (✓): Polar interaction present at the position. (x): Polar interaction not present at the position.

velopment of new drugs due to their cellular penetration. The use of CPP as peptide vectors for delivering therapeutic agents without side effects has been demonstrated (Chugh & Eudes, 2008) and according to our results, the matching SCAAP with the same properties that CPP are very high. Polarity index method (Polanco *et al.*, 2012) was initially used in the detection of SCAAP but a variation on the method (Polanco *et al.*, 2013) showed a positive identification of SCAAP-CPP with a high level of reliability (85%), as this group of peptides has proven affinity for bacterial membrane.

Polarity index method exhibits four important characteristics: (i) it uses a single physicochemical property: polarity, which is an effective SCAAP-CPP discriminator, (ii) it only uses the peptide linear sequence to identify the main pathogenic action, (iii) it determines a comprehensive profile of the peptide polar dynamics, as it considers 16 possible polar interactions and (iv) the polarity matrix is differentially weighted, thereby strengthening the SCAAP profile and minimizing false positives. The versatility of the method allows minimal changes to achieve the identification of other pathogenic groups.

Table 4. Polarity index matches by linear sequence in cell penetrating peptides.

Entry	Pubmed	Source organism	Peptide sequence	Gram (-)	Gram (+)	TI	CPP	Polarity Index	Reference
1	9923682	(KLAKKLA)2-NH2	KIAKKIAKIAKKIA	6	6	45.3			(*Alm et al., 1999*)
2	16077101	(KIAKKIA)3-NH2	KIAKKIAKIAKKIAKIAKKIA	4	4	2.8		●	(*Vasconcelos et al., 2005*)
3		(KIAKLAK)2-NH2	KIAKLAKKIAKLAK	6	6	86.2			(*Javadpour et al., 1996*)
4	16077101	(KIAKLAK)3NH2	KIAKLAKKIAKLAKKIAKLAK	4	4	2.3		●	(*Vasconcelos et al., 2005*)
5	8905231	(KALKALK)3-NH2	KALKALKKALKALKKALKALK	4	8	2.8		●	(*Kaneko et al., 1996*)
6		(KLGKKLG)3-NH2	KLGKKLGKLGKKLGKLGKKLG	4	4	98.3			(*Javadpour et al., 1996*)
7	1711035	Cecropin-A	KWKLFKKIEKVGQNIRDGIIKAGPAVAVVGQA-TQIAK	0.2	>300	1000			(*Gudmundsson et al., 1991*)
8	6309516	Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ	0.8	>0.2	500			(*Vlasak et al., 1983*)
9	2833514	Magainin-2	GIGKFLHSAKKFGKAFVGEIMNS	4	300	75	◆		*(Terry et al., 1998)*
10	1711035	CA(1–13)M(1–13)-NH2	KWKLFKKIEKVGQGIGAVLKVLTTGL	55	0.5	400			(*Gudmundsson et al., 1991*)
11	6309516	CA(1–8)M(1–18)-NH2	KWKLFKKIGIGAVLKVLTTGLPALIS	63	0.3	2000			(*Wood & Matson, 1989*)
12	2542273	Kla1	KLALKLALKAWKAALKLA	2.6	11	2.1	●	●	(*Wood & Matson, 1989*)
13		Kla2	KLALKAALKAWKAAAKLA	45	107	9.7	●	●	(*Mor et al., 1994*)
14	15001715	Kla3	KLALKAAAKAWKAAAKAA	>91	>200	2.2	●	●	(*Dietrich et al., 1994*)
15	9389475	Kla7	KAIAKSILKWIKSIAKAI	1.4	1.8	0.3			(*Klenk et al., 1997*)
16	1689726	Kla8	KALAALLKKWAKLLAALK	3	2.5	0.4	●	●	(*Sahr et al., 1990*)
17	2974085	Kla9	KLLAKAALKWLLKALKAA	1.6	1.7	0.3	●	●	(*Umesono et al., 1998*)
18		Kla10	KALKKLLAKWLAAAKALL	1.5	2	0.3	●	●	(*Lorenz et al., 1998*)
19	15229592	Kla11	KITLKLAIKAWKLALKAA	5.3	10	1.9	●	●	(*Dujon et al., 2004*)
20		Kla12	KALAKALAKLWKALAKAA	1.5	10	1.7	●	●	(*Lorenz et al., 1998*)
21	2833514	m2a	GIGKFLHSAKKFGKAFVGEIMNS	>80	428	10.7	◆		*(Terry et al., 1998)*
22	2833514	W16-m2a	GIGKFLHSAKKFGKAWVGEIMNS	>80	509	12.7			*(Terry et al., 1998)*
23	2833514	L2R11A20-m2a	GLGKFLHSAKRFGKAFVGEAMNS	75	>75	13.3			*(Terry et al., 1998)*
24	2833514	I6L15-m2a	GIGKFIHSAKKFGKLFVGEIMNS	38	38	6.8			*(Terry et al., 1998)*
25	2833514	I6A8L15I17-m2a	GIGKFIHAAKKFGKLFIGEIMNS	2.4	9.6	13.3			*(Terry et al., 1998)*
26	2833514	I6R11R14W16-m2a	GIGKFIHSAKRFGRAWVGEIMNS	37.5	>75	8.1			*(Terry et al., 1998)*
27	2833514	I6V9W12T15I17-m2a	GIGKFIHSVKKWGKTFIGEIMNS	64	2.3	24.3			*(Terry et al., 1998)*
28	2833514	100-m2a	GIAKFGKAAAHFGKKWVGELMNS	>75	700	9.3			*(Terry et al., 1998)*
29	2833514	140-m2a	GIGKFLHTLKTFGKKWVGEIMNS	13	35	2.7			*(Terry et al., 1998)*
30	2833514	160-m2a	GIGHFLHKVKSFGKSWIGEIMNS	76	82	4.3			*(Terry et al., 1998)*

Matching sequences in both SCAAP (del Rio *et al.*, 2001) and CPP (*Gautam et al., 2012*) found in APD2 (Wang & Wang, 2009). PUBMED National Center for Biotechnology information, U.S. National Library of Medicine http://blast.ncbi.nlm.nih.gob/ accessed March 20, 2013. CPP: (●) SCAAP matching CPP experimentally detected. Polarity Index: (●) SCAAP-CPP predicted by Polarity index; (●) SCAAP candidates not matching CPP predicted by polarity index method. TI: therapeutic index of a peptide, defined as the ratio between the inhibitory concentration observed in mammalian cells and the inhibitory concentration observed in bacterial cells (definition taken from del Rio (del Rio *et al.*, 2001)). (◆) Identical peptides published ~~with different TI (del Rio *et al.*, 2001) Gram (+)/Gram (-): Minimal inhibitory concentration (μg/ml) of peptide against Gram(+) and Gram (–) bacterial cells (del Rio *et al.*, 2001), Table 2A).~~

Table 5. Polarity index matches by pathogenic action.

Database	APD2	APD2	APD2	APD2	APD2	APD2	APD2	APD2	APD2	APD2	
Total hits	Gram+ ONLY	Gram– ONLY	Gram+/ Gram–	Fungi	Protists	Parasites	Insects	HIV	Cancer cells	Mammalian cells	%
Unique action	11 213	16 111	53 518	3 88	0 1	0 9	0 2	0 0	0 20	1 11	
Multiple action	19 315	18 149	179 1711	76 744	0 3	6 47	4 21	0 0	24 141	30 244	<17

Database	APD2	APD2	APD2	Uniprot	AVPpred	CPPsite	CPPsite	CPPsite			
Total hits	Chemotaxis	Virus	Sperms	H1N1	Virus	CPP Non-endocytic pathway	CPP Endocytic pathway	CPP Unknown pathway	Polarity index SCAAP	Polarity index SCAAP CPP	%
Unique action	0 0	9 60	0 0	0 35	8 60	29 93	4 22	0 0	11 30	11 13	85
Multiple action	2 39	0 0	1 9	0 0	0 0	0 0	0 0	64 405	0 0	0 0	<15

Matches found by Polarity Index method in both unique and multiple action peptide groups. Unique action: Peptides with pathogenic action against only one group. Multiple action: Peptides with pathogenic action against two or more groups. (%): Percentage hits/total peptides. Database: Name of database tested: APD2 (Wang & Wang, 2009). CPPsite (Gautam *et al.,* 2012). AVPpred (Thakur *et al.,* 2012). SCAAP set studied by Del Rio *et al.* (del Rio *et al.,* 2001). Uniprot (Magrane & Uniprot, 2011). Polarity index SCAAP: Total *SCAAP* identified by polarity index method. Polarity index SCAAP-CPP: Total *SCAAP-CPP* identified by polarity index method.

Our team has already used this method to produce an analytical classification of the APD2 database, with high efficiency results (unpublished data), indicating its potential to identify the role of a peptide.

Our group is currently working on four lines of development using this method: (i) to trace massive databases of antimicrobial peptides developing parallel versions of the source program in MPI-CUDA-F77 and MPI-CUDA-C, to be used in a computer with 4 Xeon processors in shared memory, which includes a GPU-Kepler-Nvidia cluster in GNU Linux environment, (ii) to design a new expression of the method rules to enable other researchers to generate their own rules and thus achieve the identification of other peptide groups, (iii) to develop a JAVA-version of the method source code and (iv) the synthesis and experimental verification of the three SCAAP-CPP candidates identified by the method (Table 5, entries: 2, 4 And 5).

CONCLUSIONS

In the SCAAP-CPP peptide group analytically identified here, toxicity correlates with the internment mechanism that penetrates the pathogen membrane, showing the relevance of crosschecking information in specialized databases at a regular basis, and to identify by other factors the peptides that are already known. Due to its high discriminative efficiency and low demand of computational resources, Polarity index method can be used as a first filter in peptide selection, reducing experimental trials in laboratory and making the procedure more practical. It can also be applied in the field of Proteomics to exhaustively analyze large groups of fixed length peptides, improving the understanding of how peptides are built in nature.

Availability

The test files and source program are given as "Supplementary Material".

Conflict of Interests

We declare that we do not have any financial and personal interest with other people or organizations that could inappropriately influence (bias) our work.

Author Contributions

Theoretical conception and design: CP. Computational performance: CP. Data analysis: CP, and JLS. Results discussion: CP, JLS, JACG, TB and MLS.

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