APAP, a sequence-pattern recognition approach identifies substance P as a potential apoptotic peptide

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Abstract **We have previously described a novel cancer chemotherapeutic approach based on the induction of apoptosis in targeted cells by homing pro-apoptotic peptides. In order to improve this approach we developed a computational method (approach for detecting potential apoptotic peptides, APAP) to detect short PAPs, based on the prediction of the helical content of peptides, the hydrophobic moment, and the isoelectric point. PAPs are toxic against bacteria and mitochondria, but not against mammalian cells when applied extracellularly. Among other peptides, substance P was identified as a PAP and subsequently demonstrated to be a pro-apoptotic peptide experimentally. APAP thus provides a method to detect and ultimately improve pro-apoptotic peptides for chemotherapy.** **© 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: Apoptosis; Antibacterial peptide; Bioinformatics

1. Introduction

We have previously described the finding that an antibacterial peptide, when targeted intracellularly to the angiogenic vasculature (i.e. to the endothelial cells) supplying tumors, can induce apoptosis by swelling their mitochondria [1], leading to the loss of tumor blood supply and consequent tumor regression. We named these chemotherapeutic peptides homing pro-apoptotic peptides. We designed the pro-apoptotic part of the peptides to induce endothelial cell apoptosis through mitochondrial swelling. The peptides are positively charged and the mitochondria, like bacteria, have negatively charged membranes, thus the peptides are attracted to and disrupt the mitochondrial membrane [2,3]. The initial results were obtained with a 21-residue peptide, of which the carboxy-terminal 14 amino acids represented the pro-apoptotic peptide, with the amino-terminal seven amino acids comprising the targeting peptide and a glycinylglycine bridge. The therapeutic index (TI) of the initial pro-apoptotic peptides is approximately 10. In order to increase the TI and minimize the length of these peptides, we designed a computational approach to detect short, linear and specific pro-apoptotic peptides.

Apoptosis in mammals and other eukaryotic organisms is a characteristic process of cell death, which can, among its other effects, limit the spread of viruses and other intracellular organisms [4]. For example, the difference in viral titer during baculoviral infection with and without apoptosis inhibition is 200–15 000-fold [4]. Thus apoptosis is a mechanism of defense against pathogenic infections.

Apoptosis proceeds by the activation of a group of cysteine proteases called caspases [5]. One of these, caspase-9, is activated when cytochrome *c* is released from mitochondria, which may occur with the disruption of the mitochondrial outer membrane [6]. This cytochrome *c* release in apoptotic cells may be induced by pro-apoptotic members of the Bcl-2 family, such as Bax and Bid, although the mechanism by which this is achieved is incompletely understood [7]. Nonetheless, the similarities between bacterial and mitochondrial membranes (and membrane potentials) suggested the possibility that there may be similarities between the effect of the antibacterial/pro-apoptotic peptides and pro-apoptotic Bcl-2 family members.

Antibacterial peptides in multicellular organisms are thought to serve as a defense against microbial pathogens. Originally found in invertebrates, antibacterial peptides have now been described in humans and many other organisms [2]. Among these peptides, the most well characterized are the short linear peptides (less than 40 amino acids in length) that do not contain cysteine residues. A characteristic shared by virtually all of these peptides is the presence of an amphipathic α-helical structure, which stabilizes in environments of hydrophobic nature [8] (although this helical structure has been shown not to be necessary for membrane lysis produced by a truncated form of pardaxin, an antibacterial peptide from the sole *Pardachirus marmoratus* [9]). Another characteristic shared by some of these peptides is selectivity, in that membranes from bacteria are targeted by these peptides more efficiently than mammalian plasma membranes. This selectivity is based on the complementary charge between the peptides, which are characteristically positively charged, and the negatively charged membranes of bacteria [2,3].

Structurally, these peptides typically adopt an unfolded conformation in aqueous solution. On contact with a membrane with a complementary charge, these peptides anchor to the membrane and assume an α-helical conformation. In that conformation, these peptides would either lie over the mem-

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Abbreviations: APAP, approach for detecting PAPs; PAPs, potential apoptotic peptides; SP, substance P; IP, isoelectric point; *M*, average helical hydrophobic moment; TI, therapeutic index

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brane surface in a carpet-like arrangement (in which the peptide backbone lies parallel to the membrane), or penetrate it according to the barrel–stave mechanism (in which the peptide backbone lies perpendicular to the membrane) [2]. In either case, the integrity of the membrane would be disturbed, eventually leading to membrane lysis.

In order to optimize the homing pro-apoptotic peptide approach to cancer chemotherapy by maximizing the TI (see Section 2), we have developed a theoretical approach intended to model the properties of the antibacterial peptides that present selectivity for bacteria (and thus have very low toxic effects on mammalian cells when applied extracellularly). It is our goal in this work to develop a sequence-pattern recognition approach to detect peptides that will be toxic towards mitochondria but not to mammalian cells when applied extracellularly. We refer to the peptides identified by this approach as potential apoptotic peptides (PAPs), since they may induce apoptosis by swelling mitochondria when targeted intracellularly, as previously described [1]. We refer to the approach as APAP, as an abbreviation for approach for detecting PAPs. Using APAP, we searched the SwissProt database for PAPs and among other peptides we found that substance P (SP), an extensively studied neuropeptide present in mammals, birds and fish, has all the sequence characteristics of the PAPs. Furthermore, we found that SP is capable of swelling mitochondria and inducing the cleavage of caspase-3 zymogen, a known substrate of the active form of caspase-9 in vitro. As expected, SP demonstrated very low toxicity for eukaryotic cells when applied extracellularly, in addition to displaying toxicity towards bacterial cells. These results support our sequence-pattern recognition approach to identifying new PAPs, and suggest a new role for SP in the brain.

2. Materials and methods

2.1. Sequence-pattern recognition approach

We noticed that the known antibiotic peptides fit a pattern, which includes a low likelihood of helicity in aqueous solution, a high likelihood of helicity in the presence of negatively charged membranes, and a high isoelectric point (IP). We therefore calculated the helical probability of monomeric peptides in aqueous solution (AGADIR score), the IP and the hydrophobic moment to account for the characteristics of antibacterial peptides with low toxic activity against mammalian cells. We hypothesized that these characteristics are important in determining the selectivity observed in these peptides towards bacterial membranes and bacterial-like membranes (i.e. mitochondrial membranes).

A subset of 30 antibacterial peptides previously reported in the literature was used for calculations of AGADIR scores (A) [10], IP, and average helical hydrophobic moments (M) [11], (Tables 2A and 2B). The peptide sequences of this subset are shown in Table 1.

The TI of a peptide is here defined as the ratio between the inhibitory concentration observed with mammalian cells and the inhibitory concentration observed with bacterial cells (Tables 2A and 2B). The higher the value of this ratio is, the more specific the peptide is for prokaryotic (negatively charged) membranes.

PAPs were searched for in the SwissProt database, release 38 [12], which contains a total of 80 000 protein sequences. First, all of the peptide sequences of 40 or fewer amino acids in length were extracted from this database. Then all of these sequences (2473 database entries) were used to calculate their corresponding M, IP and AGADIR scores. Protein fragments, as opposed to peptides, were not considered in this study.

2.2. Computational resources

The PEPPLOT and ISOELECTRIC programs from the GCG package (Wisconsin package version 10, USA) were used to calculate M and IP, respectively. We averaged the non-zero α-values calculated by the PEPPLOT program (see Section 2) for windows of eight residues. To calculate the AGADIR score, we used the AGADIR program, which was kindly provided by Dr. Luis Serrano at EMBL. The hydrophobicity of peptide sequences was obtained by calculating the average hydrophobicity of the sequence using the consensus scale reported by Eisenberg [11]. All these programs were run on a SGI Origin 2000 server.

2.3. Caspase-3 activation in a cell-free apoptosis system induced by SP

2.3.1. Preparation of cytoplasmic extracts. Cytoplasmic extracts were prepared as described before [18]. Briefly, non-apoptotic neuronal cells were sonicated and centrifuged at $16\,000 \times g$. This extract was made free of nuclei, mitochondria and did not self-prime.

2.3.2. Preparation of mitochondria. Rat and mouse liver mitochondria were prepared as described by Hovius et al., [13], with modifications as described previously [14]. Cultured cell mitochondria were prepared as described previously [15].

2.4. Protein electrophoresis and Western blots

Electrophoresis of proteins was carried out using either 8 or 12% SDS–polyacrylamide gels. Equal amounts of total protein were loaded per lane, and the proteins were separated at 4°C at 50 V through the stacking gel, and 90 V through the separating gel.

Western blot transfer of the proteins separated by electrophoresis was carried out at 4°C using PVDF membranes (0.2 mm) (Bio-Rad), at either 200 mA for 2 h. Blots were then blocked for 1 h in TBST (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% Tween) containing 5% non-fat dried milk. Finally, the membranes were probed with an appropriate dilution (1:500 to 1:2000) of primary antibody in TBST containing 5% non-fat dried milk for 1–4 h, depending upon the antibody.

Anti-caspase-3 antibodies from mouse, rabbit and goat were purchased from Transduction Laboratories, Inc., Upstate Biotechnology, Inc. and Santa Cruz Biotechnology, Inc., respectively.

The blots were washed three times for 1 h with TBST, followed by incubation in a peroxidase-coupled secondary antibody for 1 h in TBST containing 5% non-fat dried milk. The mouse, human, and rabbit peroxidase-coupled secondary antibodies were from Amersham. Enhanced chemiluminescence detection of the proteins was carried out using Hyperfilm ECL (Amersham), and with Pierce Super-Signal Substrate Western Blotting reagents, or Amersham ECL reagents.

2.5. Mitochondrial swelling assays

Rat liver mitochondria were prepared as described above. The peptide concentrations used to swell mitochondria were 50 μM L-SP, 10 μM D-(LSLARLATARLAI) (negative control), or 200 μM Ca^{+2} (positive control). The swelling was quantified by measuring the optical absorbance at 540 nm.

2.6. Activity of SP on fibroblasts

10^4 human embryonic kidney 293 cells per well were seeded into a 96-well plate. After 20 h, different aqueous dilutions (Fig. 2B) of SP (Sigma, USA), C31 and a peptide used as a control were added to the culture and the cell death was quantified by trypan blue exclusion 48 h later.

2.7. Toxicity of SP for bacterial cells

DH5α *Escherichia coli* cells were grown overnight as a pre-inoculum for the bacterial culture used in this assay. When the cells were at the end of their log phase (optical density at 600 nm of 0.8–1.0), 1 μl was used to inoculate 5 ml. Such dilution produced initial concentrations of bacteria capable of forming 10^5–10^6 colonies per ml in LB plates at 37°C, that is 10^5–10^6 colony forming units. All the bacterial cultures used in these experiments were grown in LB at 37°C. The concentration of SP required to inhibit the cell growth by 60% was determined by following bacterial growth in LB liquid in the presence of varying concentration of the peptide: 0, 1, 10, 20, 50, 125, and 250 μM. Sterilized 96-well plates of polystyrene with flat bottom and low evaporation lid (Costar, USA) were used, in a final volume of 100 μl: 50 μl of LB containing 10^5–10^6 colony forming units, and 50 μl of LB with a 2-fold dilution of the peptide. A 10 mM stock solution of the peptide was prepared with 5 mg of SP in 371 μl of water. Inhibition of growth was detected by measuring optical density at 600 nm with a microplate spectrophotometer SPECTRAmax (Molecular Devices,

USA) at varying times: 0, 3, 5, 6, 7 and 8 h. Each IC_{60} was determined from at least two independent experiments performed in triplicate. Additionally, the colonies formed from each experiment were counted in LB plates at 0 and 8 h of growth.

3. Results

The antibacterial peptides analyzed and biophysical properties previously determined are presented in Tables 1 and 2A, respectively.

In order to reproduce these biophysical properties, we calculated three scores from the sequences of these peptides. Table 2A shows a subset of selected antibacterial peptide sequences (see Section 2) and the corresponding experimental values for helix formation in water and in hydrophobic environments, antibacterial activity and cytotoxic activities against mammalian cells. Table 2B shows the corresponding calculated values for *M*, IP, *A* and the TI. We observed that the antibacterial peptides presented in Table 1 are more potent against G(−) (MIC = 17.3 μg/ml on average) bacteria than to G(+) (MIC = 44.3 μg/ml), and we used the G(−) values as a reference for the TI.

Peptide sequences with values ranging from $0.4 < M < 0.6$, $A < 10.0$ and $10.8 < IP < 11.7$, were found to have the highest TI (highest specificity for bacteria) (Table 2B). These parameters were therefore hypothesized to be the signature of the PAPs. Searching for PAPs in the SwissProt database led us to identify 14 PAPs (Table 3). Two of these peptides have previously been characterized with respect to their toxicity against bacteria and mammalian cells, and in both cases a greater toxicity towards bacterial cells was observed (Table 3).

3.1. Swelling of mitochondria and activation of caspase-3 by SP

One of the PAPs identified, SP, was tested for its ability to swell mitochondria and induce caspase-3 activation in a cell-free system. This system was developed previously in our group to simulate neuronal apoptosis (see Section 2 and [14]). We observed that SP induces the swelling of mitochondria at 50 μM in our system (data not shown). At such concentration, SP was capable of releasing cytochrome *c* from mitochondria and activating caspase-3 (Fig. 1). In contrast, a peptide chosen as negative control (see Section 2) which did not present the properties of PAPs (data not shown) did not display any observable effect on mitochondria (Fig. 1).

3.2. TI of SP

The toxicity of SP against bacteria was tested and compared to the effect of SP on fibroblasts when applied extracellularly. SP was able to reduce the growth of *E. coli* cells with an IC_{60} of 10 μM (Fig. 2B). By comparison, the negative control peptide did not have any toxicity against bacteria. In contrast, Fig. 2A shows that SP did not affect the growth of fibroblasts when applied extracellularly even at a concentration of 1 mM. These results indicate that SP has a $TI > 100$. Additionally, a peptide from the protein APP (the last 31 amino acids in APP, referred as C31) known to induce apoptosis when expressed intracellularly [16] was tested for its toxicity against bacteria and mammalian cells. This peptide did not present the properties (IP, *M*, *A* scores) of PAPs (data not shown). C31 did not present any observable toxicity against bacterial or mammalian cells when applied extracellularly (Fig. 2A,B).

Table 1
Peptide sequences of a subset of antibacterial peptides

Peptide name	Peptide sequence
(KIAKKIA)2NH2	KIAKKIAKIAKKIA-NH2
(KIAKKIA)3NH2	KIAKKIAKIAKKIAKIAKKIA-NH2
(KIAKLAK)2NH2	KIAKLAKKIAKLAK-NH2
(KIAKLAK)3NH2	KIAKLAKKIAKLAKKIAKLAK-NH2
(KALKALK)3NH2	KALKALKKALKALKKALKALK-NH2
(KLGKKLG)3NH2	KLGKKLGKLGKKLGKLGKKLG-NH2
CecropinA	KWKLFKKIEKVGQNIRDGIIKAGPAVAVVGQATQIAK-NH2
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ-NH2
Magainin 2	GIGKFLHSAKKFGKAFVGEIMNS-NH2
CA(1–13)M(1–13)NH2	KWKLFKKIEKVGQGIGAVLKVLTTGL-NH2
CA(1–8)M(1–18)NH2	KWKLFKKIGIGAVLKVLTTGLPALIS-NH2
Kla1	KLALKLALKAWKAALKLA-NH2
Kla2	KLALKAALKAWKAAAKLA-NH2
Kla3	KLALKAAAKAWKAAAKAA-NH2
Kla7	KAIAKSILKWIKSIAKAI-NH2
Kla8	KALAALLKKWAKLLAALK-NH2
Kla9	KLLAKAALKWLLKALKAA-NH2
Kla10	KALKKLLAKWLAAAKALL-NH2
Kla11	KITLKLAIKAWKLALKAA-NH2
Kla12	KALAKALAKLWKALAKAA-NH2
m2a	GIGKFLHSAKKFGKAFVGEIMNS-NH2
W16-m2a	GIGKFLHSAKKFGKAWVGEIMNS-NH2
L2R11A20-m2a	GLGKFLHSAKRFGKAFVGEAMNS-NH2
I6L15-m2a	GIGKFIHSAKKFGKLFVGEIMNS-NH2
I6A8L15I17-m2a	GIGKFIHAAKKFGKLFIGEIMNS-NH2
I6R11R14W16-m2a	GIGKFIHSAKRFGRAWVGEIMNS-NH2
I6V9W12T15I17-m2a	GIGKFIHSVKKWGKTFIGEIMNS-NH2
100-m2a	GIAKFGKAAAHFGKKWVGELMNS-NH2
140-m2a	GIGKFLHTLKTFGKKWVGEIMNS-NH2
160-m2a	GIGHFLHKVKSFGKSWIGEIMNS-NH2

The amino acids in the peptide sequence are represented in a one-letter code.

Table 2A
Observed characteristics of a subset of antibacterial peptides

Peptide	CD Water	Observed lipid	Antibacterial Gram(−)	Activity Gram(+)	Cytotoxicity	Reference
(KLAKKLA)2NH2	<5	24	6	6	>272	[8]
(KLAKKLA)3NH2	<5	79	4	4	>11	[8]
(KLAKLAK)2NH2	<5	37	6	6	>517	[8]
(KLAKLAK)3NH2	<5	79	4	4	>9	[8]
(KALKALK)3NH2	<5	67	4	8	11	[8]
(KLGKKLG)3NH2	<5	33	4	4	>393	[8]
Cecropin A	0	75	0.2	>300	>200	[24]
Melittin	0	75	0.8	>0.2	>400	[24]
Magainin 2	0	44	4	300	300	[24]
CA(1–13)M(1–13)NH2	0	55	0.5	2	>200	[24]
CA(1–8)M(1–18)NH2	0	63	0.3	1	>600	[24]
Kla1	ND	73	5.2	2.6	11	[25]
Kla2	ND	68	11	45	107	[25]
Kla3	ND	59	91	>91	>200	[25]
Kla7	ND	70	5.6	1.4	1.8	[25]
Kla8	ND	62	5.8	3	2.5	[25]
Kla9	ND	55	6.2	1.6	1.7	[25]
Kla10	ND	62	6.1	1.5	2	[25]
Kla11	ND	69	5.3	5.3	10	[25]
Kla12	ND	67	6	1.5	10	[25]
m2a	ND	57	40	>80	428	[25]
w16-m2a	ND	57	40	>80	509	[25]
l2r11a20m2a	ND	45	75	>75	>100	[25]
i6l15-m2a	ND	57	38	38	260	[25]
i6a8l15i17m2a	ND	61	2.4	9.6	32	[25]
i6r11r14w16m2a	ND	52	37.5	>75	303	[25]
i6v9w12t15i17-m2a	ND	64	2.3	18	56	[25]
100-m2a	ND	48	75	>75	700	[25]
140-m2a	ND	75	13	13	35	[25]
160-m2a	ND	54	19	76	82	[25]

Peptide: see Table 1 for the amino acid composition for each peptide described in this table. CD Observed in water or lipid: percent of α-helical secondary structure determined by circular dichroism. Antibacterial activity G(+) or G(−): the minimal inhibitory concentration (μg/ml) for each peptide against Gram(+) and Gram(−) bacterial cells. Cytotoxicity: the concentration (μg/ml) required for inhibiting the growth of mammalian cells, usually red blood cells or fibroblasts.

4. Discussion

In order to optimize our homing pro-apoptotic approach to target and kill angiogenic endothelial cells supplying cancer cells, we have developed APAP, an approach to detect PAPs. APAP was originally developed to overcome the problems of toxicity and synthesis associated with our chemotherapeutic approach [1]. Positively charged PAPs, which are non-toxic outside the cell, are targeted to tumor vasculature by a fusion with a peptide that recognizes a receptor on the cell surface [17,18] and consequently internalized where they disrupt the negatively charged mitochondria, thereby exerting their pro-apoptotic effect.

We calculated the amphipathicity, IP and AGADIR scores for a subset of 30 different antibacterial peptides. The values grouping those peptides with the highest TI were considered the signature of PAPs. We searched for PAPs that resemble antibacterial ones, based on the posited relationship between mitochondrial-dependent apoptosis mechanisms and antibacterial activity. APAP provided us with a tool to identify PAPs independently of any sequence similarity with other known antibacterial or pro-apoptotic peptides. Additionally, APAP allowed us to search sequence databases systematically.

We calculated the amphipathicity and IP because amphipathic peptides are known to be membrane-associated [11], and the selectivity for recognizing bacterial-like membranes depends on the composition of the membranes [2,3]. Additionally, it has been previously recognized that hydrophobic peptides display both antibacterial activity and toxicity against mammalian cells [19] (i.e. non-selective toxicity), thus PAPs would be expected not to be simply highly hydrophobic peptides. We observed that in our group of peptides (Table 1), all of the peptides but one were hydrophilic, constituting an appropriate group of peptides from which to select PAPs. It has been shown previously that antibacterial peptides with lower hydrophobicity display higher specificity towards Gram-negative bacteria [20]. In agreement with this notion, all the peptides analyzed in our study presented higher specificity towards G(−) bacteria as expressed by the TI values (Tables 2A and 2B).

Alternatively, the propensity to form soluble structures in water (expressed by the propensity to form secondary struc-

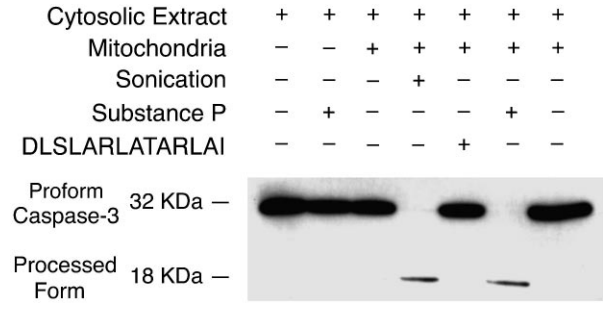

Fig. 1. Pro-apoptotic activity of SP. The release of cytochrome *c* from mitochondria and the processing of caspase-3 into the active form are shown for SP and controls (sonication, the detergent Triton X-100 and a non-toxic peptide DLSLARLATARLAI).

Table 2B
Calculated characteristics of a subset of antibacterial peptides

Peptide	*A*	*M*	IP	⟨*H*⟩	TI
(KLAKKLA)2NH2	4.5	0.48	11.5	−0.249	45.3
(KLAKKLA)3NH2	16.2	0.48	11.7	−0.249	2.8
(KLAKLAK)2NH2	5.1	0.48	11.5	−0.249	86.2
(KLAKLAK)3NH2	17.2	0.48	11.7	−0.249	2.3
(KALKALK)3NH2	16.6	0.48	11.7	−0.249	2.8
(KLGKKLG)3NH2	1.1	0.49	11.7	−0.274	98.3
Cecropin A	1.2	0.44	11.2	−0.123	1000.0
Melittin	3.1	0.46	12.6	−0.83	500.0
Magainin 2	0.8	0.56	10.8	−0.036	75.0
CA(1–13)M(1–13)NH2	1.1	0.53	11.1	−0.46	400
CA(1–8)M(1–18)NH2	1.3	0.43	11.4	0.065	2000
Kla1	13.4	0.16	11.4	−0.025	2.1
Kla2	10.6	0.30	11.4	−0.056	9.7
Kla3	7.2	0.17	11.4	−0.087	2.2
Kla7	2.4	0.53	11.4	−0.026	0.3
Kla8	49	0.51	11.4	−0.025	0.4
Kla9	18	0.38	11.4	−0.025	0.3
Kla10	23.5	0.45	11.4	−0.025	0.3
Kla11	14.8	0.16	11.4	−0.027	1.9
Kla12	19.5	0.49	11.4	−0.056	1.7
m2a	0.8	0.56	10.8	−0.036	10.7
w16-m2a	0.9	0.49	10.8	−0.046	12.7
12r11a20-m2a	0.9	0.51	11.1	−0.094	13.3
i6l15-m2a	0.6	0.54	10.8	−0.095	6.8
i6a8l15i17-m2a	1.1	0.55	10.8	0.016	13.3
i6r11r14w16-m2a	0.8	0.48	11.7	−0.095	8.1
i6v9w12t15i17-m2a	0.7	0.56	10.8	−0.035	24.3
100-m2a	1.1	0.46	10.8	−0.045	9.3
140-m2a	0.9	0.57	10.8	−0.049	2.7
160-m2a	0.7	0.57	10.5	−0.017	4.3

Peptide: see Table 1 for the amino acid composition for each peptide described in this table. *A*: AGADIR score. *M*: average helical hydrophobic moment. IP: estimated isoelectric point. ⟨*H*⟩: averaged hydrophobicity. TI: calculated therapeutic index.

tures in water, AGADIR score) was used in our approach. Since hydrophobicity and the propensity to form soluble structures in water are inversely related, it is expected that hydrophobic sequences will display a low AGADIR score. The inverse is not necessarily true, though; that is, peptide sequences with low AGADIR scores are not necessarily hydrophobic. Interestingly, PAPs tend to be hydrophilic with low AGADIR scores (Tables 2B and 3).

The peptides used to define the parameters of the PAPs (Table 1) are mostly synthetic peptides, with the exception of three natural peptides (magainin, cecropin A and melittin). None of these three natural peptides in Table 1 were detected in our analysis because they were deposited in the SwissProt database in their mature form. In this form, they were longer than the cut-off value used to define the peptide database analyzed in this study (see Section 2 for a description of the peptide database used in this study). Alternatively, two cecropins (cec4_bommo, cecb_antpe) and two other natural antibacterial peptides (crbl_vescr, dms3_physa) were found in our search. In agreement with our predictions, these antibacterial peptides have been reported to have TIs similar to PAPs (Table 3). As further evidence of the validity of our approach, we tested two peptides, C31 and a control, that did not match the IP, *M* and *A* scores of PAPs (Fig. 2). The C31 peptide has

Table 3
PAPs in the SwissProt database

SwissProt name	*A*	*M*	IP	⟨*H*⟩	Length	Antibacterial activity Gram(−)	Cytotoxicity	Reference
Bol1_megpe	7.9	0.52	11.1	0.058	17			
Cec4_bommo	0.5	0.44	11.3	−0.097	35			
Cecb_antpe	0.5	0.43	11.5	−0.132	35			
Crbl_vescr	0.7	0.50	11.6	0.144	13	15	> 120	[24]
Dms3_physa	1.7	0.44	11.1	−0.024	30	2.5	80	[26]
Grar_ranru	0.04	0.53	11.6	−0.084	12			
Ranr_ranru	1.3	0.44	11.6	−0.239	17			
Npf_arttr	4.3	0.45	10.9	−0.297	36			
sp5m_bacsu	2.5	0.55	11.4	−0.095	26			
Stp_bpt4	1.7	0.43	11.1	−0.278	26			
Tkna_gadmo	0.03	0.48	11.6	−0.190	11			
Tkna_horse	0.03	0.51	11.6	−0.201	11			
Tkna_oncmy	0.01	0.49	11.6	−0.175	11			
Tkna_scyca	0.03	0.49	11.6	−0.124	11			

SwissProt name: the accession name in the SwissProt database for that particular peptide. *A*: AGADIR score. *M*: average helical hydrophobic moment. IP: calculated isoelectric point. ⟨*H*⟩: averaged hydrophobicity. Cytotoxicity: the concentration (μg/ml) required for inhibiting the growth of mammalian cells, usually red blood cells or fibroblasts.

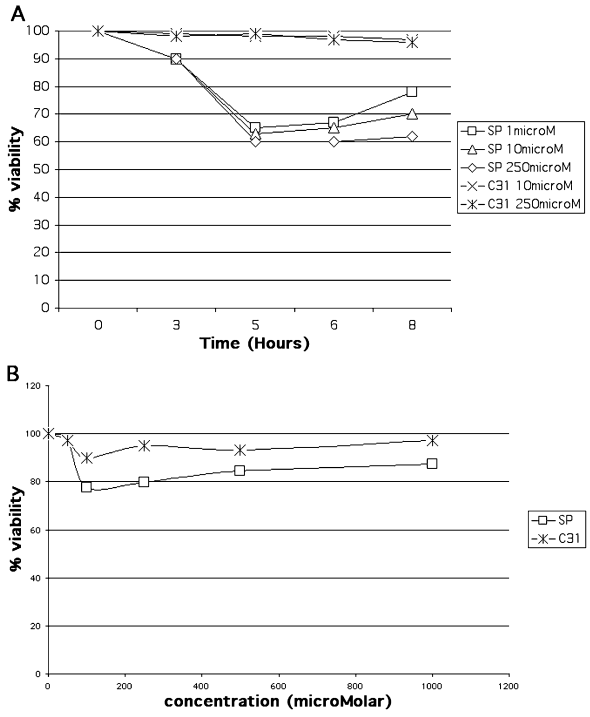

Fig. 2. Selective toxicity of SP on bacteria. The effect of SP and C31 on cell viability was measured on fibroblast cells (A) and bacteria cells (B). The viability is reported relative to a control (peptide DLSLARLATARLAI).

been shown to induce apoptosis by an unknown mechanism [16], so we considered it an interesting target for our study since we might provide some hints on the mechanism of action of C31 in addition to testing our approach. We found that none of these peptides is toxic to bacterial or mammalian cells when applied extracellularly thus confirming our predictions. Based on these results we propose that C31 may induce apoptosis by a different mechanism than PAPs.

In total, 14 sequences were identified as PAPs in the SwissProt database (see Section 2). These 14 peptides can be placed into four different groups based on their known function; i.e. antibacterial peptides, neuropeptides, mast cell degranulating peptides and protein–protein interacting peptides. Two out of these four groups, antibacterial peptides and neuropeptides, represent more than 80% of the total (Table 3). Neuropeptides appear to be over-represented since there were only 48 neuropeptides in the original pool of 2473 peptides in the SwissProt database.

The special need for antibacterial peptides in the mammalian brain has been pointed out previously [21], since these may represent a more immediate line of control for bacterial infection than the immune system (which has a restricted access to the brain). Considering the properties of PAPs, our findings suggest that some previously identified neuropeptides may have antibacterial activity.

Among the neuropeptides identified as PAPs (Table 3), four were homologs of SP: tkna_gadmo, tkna_horse, tkna_oncmy, and tkna_scyca. SP belongs to the tachykinin family. Tachykinins are synthesized as larger protein precursors (usually more than 40 amino acids in length) that are enzymatically converted to their mature forms [22]. In our original search, we were able to detect only those recorded in the SwissProt database in the active form. Analyzing all of the tachykinins deposited in the database (precursors and active forms), we found that 10 out of 61 were predicted to be PAPs (data not shown). Notably, these 10 were SP peptides from different species.

SP is known to form an α-helical structure in hydrophobic environments but not in aqueous solution [23], while it has a positive charge distribution over its sequence, supporting the finding that SP is a PAP. Therefore, the neuropeptide SP was tested for its preference for mitochondria-like membranes. The results presented in this work support our predictions that SP is a PAP. However, we did not observe a complete inhibition of *E. coli* growth, probably because of its well known short half-life in solution (minutes), while our experiments lasted for 8 h. Another possibility is that SP only displays a bacteriostatic activity, since the toxicity displayed by SP on bacterial cells was not markedly affected by the concentration of SP, as in the case of antibacterial peptides.

In developing APAP we focused on the characteristics that define selectivity rather than efficiency to kill bacteria. Therefore, it is not surprising that SP demonstrated bacteriostatic, but not bactericidal, activity. It is noteworthy that SP and most of the antibacterial peptides analyzed in this study (Table 1) are active in the low micromolar concentration range, and that SP is only 11 amino acids long. However, SP was toxic at higher concentrations than the antibacterial peptides in Table 1. We are currently working to use APAP to design more effective antibacterial peptides with higher TI values.

In conclusion, we have described a computational approach, APAP, to identify PAPs. These peptides display selectivity towards bacteria and mitochondria, with little toxic effect on eukaryotic cells when applied extracellularly, thus providing the basis for a new generation of drugs that can be present in the body without toxic effect unless they are taken in by targeted cells as we have shown previously [1]. From a public database, the approach detected mostly antibacterial peptides and neuropeptides suggesting that these neuropeptides may be the first reported with antibacterial activity. In agreement with this idea, we reported that SP is a PAP with a TI $>$ 100. We speculate that these activities have been present in SP during the course of evolution of the tachykinins, which would support the possibility of a biological significance for these findings. APAP provides a method to detect and ultimately improve pro-apoptotic peptides for chemotherapy.

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