PLOS ONE

RESEARCH ARTICLE

Bioprospecting the American Alligator (*Alligator mississippiensis*) Host Defense Peptidome

Barney M. Bishop[1]*, Melanie L. Juba[1], Megan C. Devine[1], Stephanie M. Barksdale[2], Carlos Alberto Rodriguez[1], Myung C. Chung[2], Paul S. Russo[3], Kent A. Vliet[4], Joel M. Schnur[5], Monique L. van Hoek[2,6]

1 Department of Chemistry and Biochemistry, George Mason University, Fairfax, Virginia, United States of America, **2** School of Systems Biology, George Mason University, Manassas, Virginia, United States of America, **3** Center for Applied Proteomics and Molecular Medicine, George Mason University, Manassas, Virginia, United States of America, **4** Department of Biology, University of Florida, Gainesville, Florida, United States of America, **5** College of Science, George Mason University, Fairfax, Virginia, United States of America, **6** National Center for Biodefense and Infectious Diseases, George Mason University, Manassas, Virginia, United States of America

* bbishop1@gmu.edu

OPEN ACCESS

Citation: Bishop BM, Juba ML, Devine MC, Barksdale SM, Rodriguez CA, Chung MC, et al. (2015) Bioprospecting the American Alligator (*Alligator mississippiensis*) Host Defense Peptidome. PLoS ONE 10(2): e0117394. doi:10.1371/journal.pone.0117394

Academic Editor: Jürgen Harder, University Hospital Schleswig-Holstein, Campus Kiel, GERMANY

Received: October 15, 2014

Accepted: December 22, 2014

Published: February 11, 2015

Copyright: © 2015 Bishop et al. This is an open access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: The authors gratefully acknowledge the Defense Threat Reduction Agency (http://www.dtra.mil/) HDTRA1-12-C-0039 and the George Mason University College of Science (http://www.gmu.edu) for support. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Abstract

Cationic antimicrobial peptides and their therapeutic potential have garnered growing interest because of the proliferation of bacterial resistance. However, the discovery of new antimicrobial peptides from animals has proven challenging due to the limitations associated with conventional biochemical purification and difficulties in predicting active peptides from genomic sequences, if known. As an example, no antimicrobial peptides have been identified from the American alligator, *Alligator mississippiensis*, although their serum is antimicrobial. We have developed a novel approach for the discovery of new antimicrobial peptides from these animals, one that capitalizes on their fundamental and conserved physico-chemical properties. This sample-agnostic process employs custom-made functionalized hydrogel microparticles to harvest cationic peptides from biological samples, followed by *de novo* sequencing of captured peptides, eliminating the need to isolate individual peptides. After evaluation of the peptide sequences using a combination of rational and web-based bioinformatic analyses, forty-five potential antimicrobial peptides were identified, and eight of these peptides were selected to be chemically synthesized and evaluated. The successful identification of multiple novel peptides, exhibiting antibacterial properties, from *Alligator mississippiensis* plasma demonstrates the potential of this innovative discovery process in identifying potential new host defense peptides.

Introduction

There has been a growing interest in cationic antimicrobial peptides (CAMPs) as a potential source of new therapeutics with which to address the growing problem of bacterial antibiotic

resistance [1, 2]. Nature provides a prescreened library of peptides that has been selected over millions of years of evolution for their ability to defend against infection under physiological conditions. The American alligator (*Alligator mississippiensis*) and other crocodilians are evolutionarily ancient animals whose plasma and leukocyte extracts have been shown to exhibit antimicrobial activity [3–5]. This antimicrobial potency may be attributable at least in part to the presence of CAMPs in the alligator plasma and extracts. Cationic antimicrobial peptides have been shown to be capable of exerting significant antibacterial effects, and they figure prominently in the innate immunity of vertebrates and other higher organisms. Despite the interest in the American alligator and the antimicrobial peptides that they may produce, no CAMPs have been identified from their blood or tissues to date [3, 6].

The discovery and identification of novel CAMPs from animals has proven challenging using conventional proteomics tools. Methods used to fractionate and isolate peptides are labor-intensive, can result in sample and activity loss, and are unable to detect low-abundance peptides. To address these limitations, prior efforts to identify crocodilian antimicrobial peptides have resorted to using very large sample volumes [5, 7], which can be problematic if the animals are endangered or sample size is limiting. Further complicating matters, the high sequence and structural diversity of CAMPs presents an impediment to traditional bottom-up proteomics mass spectrometry methods, which employ proteolytic digestion and database searches to facilitate peptide sequence determination. Subjecting samples to proteolytic digestion in this manner destroys information regarding the original native, intact peptide sequences. To overcome these challenges, we have employed a multidisciplinary strategy that draws from protein biophysics, peptide chemistry, nanomaterials, advanced mass spectrometry techniques, and microbiology.

We report here the development of a new bioprospecting particle-assisted proteomics approach to antimicrobial peptide discovery, which builds upon recent advances in proteomics and biomarker discovery [8, 9]. It utilizes a novel approach for extracting peptides, including CAMPs, from very small sample volumes (e.g. 100μl) followed by analysis of the harvested peptides using advanced middle-down mass spectrometry techniques and *de novo* peptide sequencing to identify CAMPs that may be present (Fig. 1). We have applied this process to the discovery of novel CAMPs from plasma from the American alligator. It employs custom-made functionalized hydrogel microparticles to harvest CAMPs and CAMP-like peptides in their

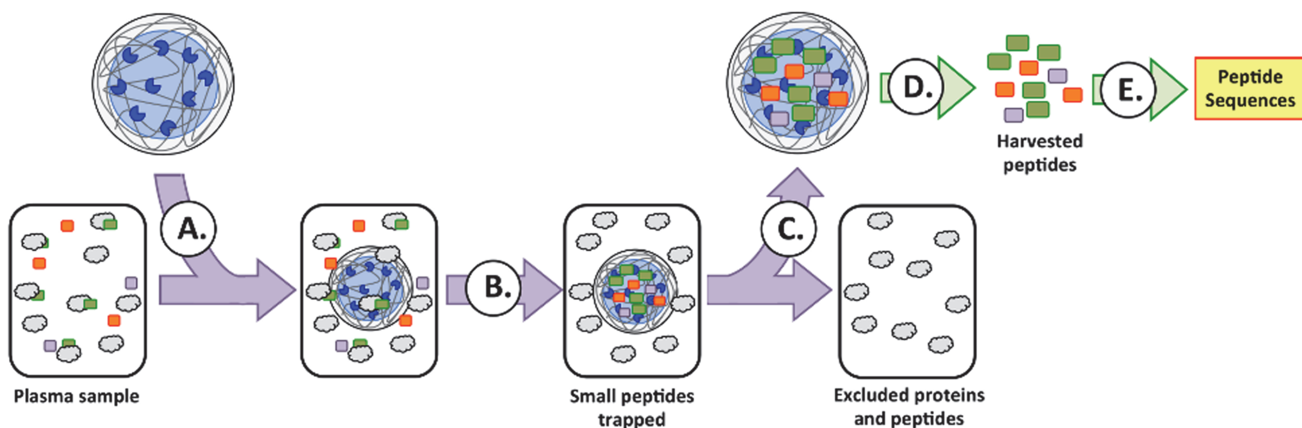

Fig 1. Bioprospecting Approach to CAMP discovery. (A) Hydrogel microparticles are introduced into the plasma sample. (B) The particles capture small cationic peptides which are present in the sample, while excluding high molecular weight proteins. (C) The particles are then recovered, (D) captured low molecular weight peptides are eluted from the particles and (E) analyzed by high-resolution MS/MS.

doi:10.1371/journal.pone.0117394.g001

native form from biological samples, agnostic to source, based on their physico-chemical properties. Mass spectral analysis of the harvested intact peptides using an Orbitrap Elite mass spectrometer equipped with electron transfer dissociation (ETD) is used to determine their sequences in a *de novo* manner. The sequences are compared to available genomic and proteomic information in order to confirm, complete and correct the *de novo* peptide sequences. Furthermore, all sequences are ultimately manually verified, especially those for which no genomic information is available. Two selection criteria are applied to down-select peptides for further testing. In one approach, potential CAMPs are identified from the peptide sequences using web-based prediction tools (*CAMP* database, *AntiBP2* and *APD2*) [10–12]. In the other approach, prospective CAMPs are selected through rational analysis of the peptide sequences, focusing primarily on size, charge, and sequence similarity with other potential CAMPs. In our analysis of alligator plasma, we identified forty-five potential CAMPs. In this process, the particle harvest is used for peptide discovery, not purification. For further testing, identified cationic peptides must be synthesized and their antimicrobial activities determined. In order to provide an initial assessment of the rational and web-based CAMP prediction methods, eight CAMP candidates were synthesized and evaluated for activity. Four of the eight peptides were selected because they had physico-chemical properties consistent with known CAMPS, specifically being highly cationic with nominal charges ranging from +4 to +5 ($APOC1_{64–88}$, $APOC1_{67–88}$, $A1P_{394–428}$, ASAP130LP). The next two peptides were chosen because the predictive algorithms indicated a high probability that they would be antimicrobial despite having lower net cationic charge of +2 and +3 (AVTG2LP and $NOTS_{17–38}$). The remaining two peptides were selected because they exhibited overlap between both selection criteria, having both positive CAMP prediction scores and good physico-chemical properties ($FGG_{398–413}$ and $FGG_{401–413}$). Evaluation of these eight peptides led to the identification of five novel alligator peptides that demonstrate antimicrobial activity, $APOC1_{64–88}$, $APOC1_{67–88}$, $A1P_{394–428}$, $FGG_{398–413}$ and $FGG_{401–413}$.

Results

The first step in the bioprospecting process used to identify novel alligator CAMPs employed hydrogel microparticles based on cross-linked N-isopropylacrylamide copolymer frameworks, which are central to the CAMP discovery process [8, 9]. Harvesting was performed using a 50:50 combination of two types of particles, one incorporating acrylic acid as its affinity bait and the other combining acrylic acid and 2-acrylamido-2-methyl-propanesulfonic acid as baits. These particles enable multidimensional separation of targeted peptides from other proteins and peptides present in the samples. Negatively charged acidic groups, such as carboxylic acids and sulfonic acids, provide affinity baits for the capture of cationic peptides and proteins. At the same time, the cross-linking of the polymer scaffold excludes larger peptides and proteins, while allowing low molecular weight peptides access to affinity baits residing in the particle interior. Thus, the particles simultaneously combine elements of cation exchange and size-exclusion chromatography when capturing peptides and proteins from complex biological samples, favoring peptides with physico-chemical properties similar to those of CAMPs. Additionally, hydrogel particles have been shown to protect captured labile biomolecules from degradation during the harvesting process [9].

In our first experiment, we sought to establish that the particles harvested known CAMPs from alligator plasma. Due to the limited available information regarding the CAMPs that may be present in alligator plasma, the ability of the hydrogel particle combination to capture CAMPs from 100 μL of commercial alligator plasma was evaluated using a mixture of known peptides (60 pmol each) representing different CAMP classes: buforin (histone-derived), SMAP-29 (helical), and indolicidin (linear Trp/Arg/Pro-rich). The model-peptide sample was

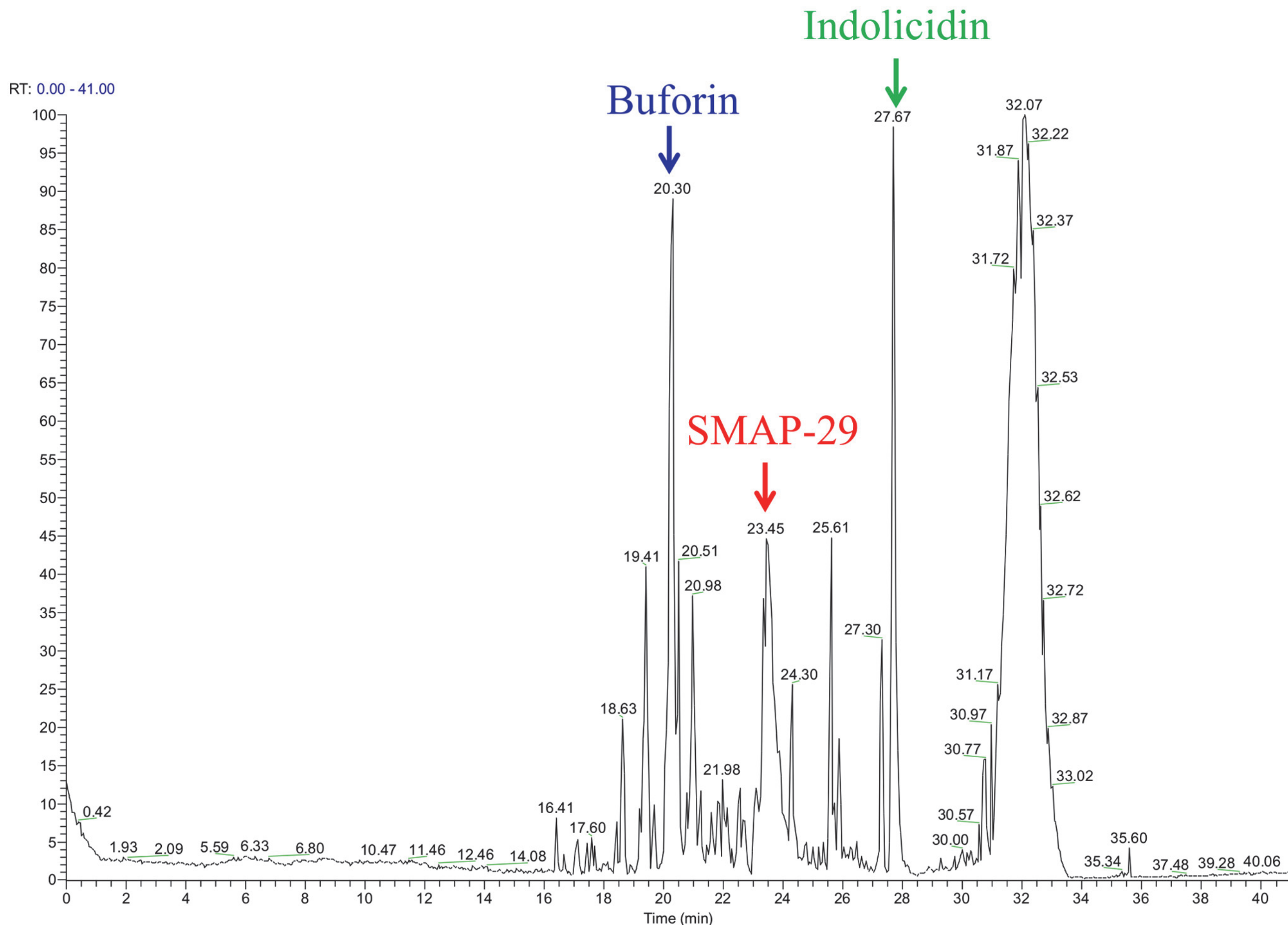

Fig 2. Mass Spectral Verification of CAMP Harvesting by Hydrogel Particles. Mass spectra of particle eluents following harvesting from of American alligator plasma spiked with 3 known CAMPs (Buforin, SMAP-29 and Indolicidin). The CAMP peaks in the spectra are identified and labeled with their corresponding identity.

doi:10.1371/journal.pone.0117394.g002

diluted into an aqueous suspension of hydrogel particles, and the harvest mixture was then incubated for 18 hours at room temperature. The particles were recovered by centrifugation and washed to remove unbound, excluded peptides and proteins. Captured peptides were eluted from the washed particles and analyzed by mass spectrometry, which revealed that the particles had effectively harvested the model CAMPs from the plasma along with alligator plasma peptides (Fig. 2). Buforin harvesting was confirmed by the presence of the $(M+4H)^{+4}$ ion at 609.4 m/z, the $(M+5H)^{+5}$ ion at 487.7 m/z and the $(M+6H)^{+6}$ ion at 406.6 m/z. SMAP-29 capture was identified by the $(M+5H)^{+5}$, $(M+6H)^{+6}$ and $(M+7H)^{+7}$ at 651.8 m/z, 543.3 m/z and 465.9 m/z, respectively. The harvesting of indolicidin was verified by the presence of the $(M+3H)^{+3}$ ion at 636.0 m/z and the $(M+4H)^{+4}$ ion at 477.3 m/z, thus demonstrating the ability of the particles to capture known CAMPs and CAMP-like peptides from plasma.

With the ability of the hydrogel particles to capture known CAMPs from plasma established, the harvesting process was then applied to stimulated alligator plasma for the identification of novel crocodilian CAMPs. Prior to harvesting, alligator blood was treated with ionomycin to stimulate peptide release into the plasma from heterophils. Ionomycin, which is a calcium ionophore, has been demonstrated to trigger the release of hCAP18 (the human cathelicidin LL-37 precursor) from neutrophil granules [13]. Following stimulation, the plasma

C [+2] ions 241 291 369 426 519 593 636 701 769 843 907 971 1021 1085 1149 1213 1270 1334 1391 1442 1515
C ions 165 252 353 481 582 738 852 1038 1185 1272 1401 1538 1685 1813 1942 2041 2169 2298 2426 2539 2667 2782 2883 3030
F S T K T R N W F S E H F K K V K E K L K D T F A
Z ions 2939 2852 2751 2623 2522 2366 2252 2066 1919 1832 1702 1565 1418 1290 1162 1063 935 806 678 565 437 322 221 74
Z [+2] ions 1470 1426 1376 1312 1261 1183 1126 1033 960 916 851 783 709 645 581 532 468 403 339 283

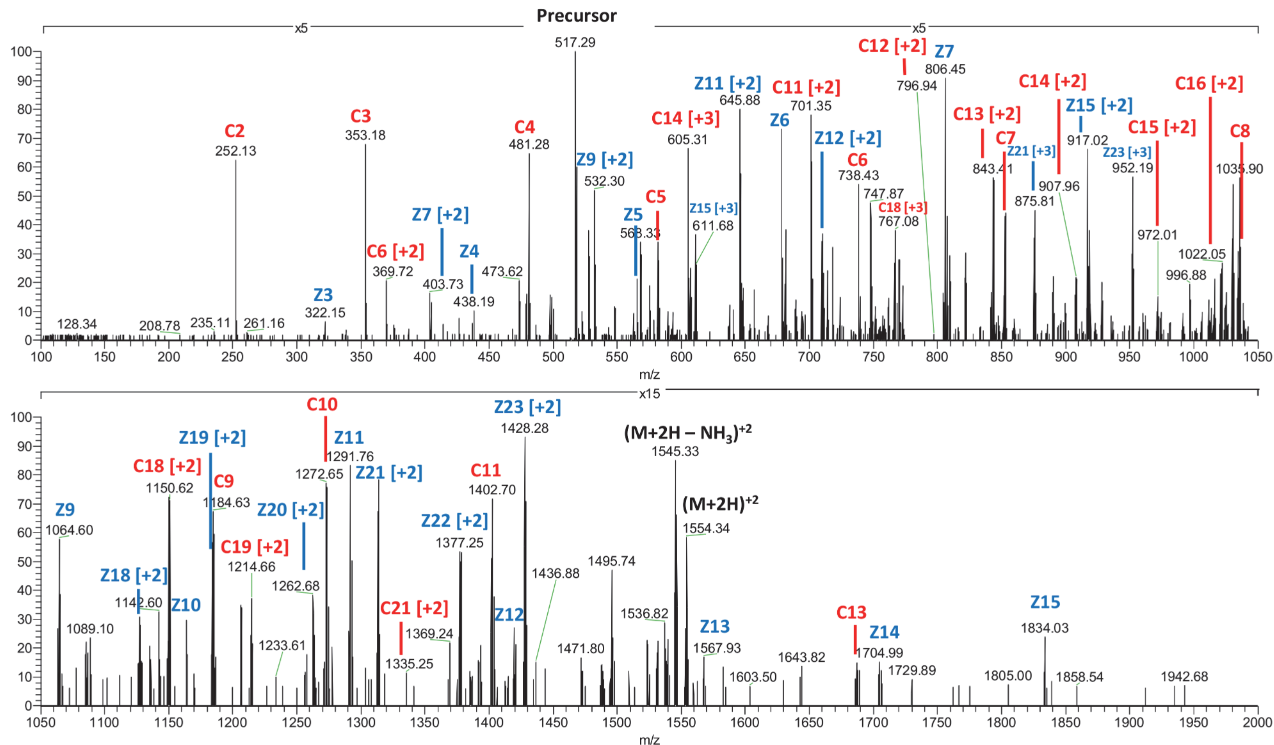

Fig 3. ETD Mass Spectrum for APOC1$_{64–88}$. ETD mass spectrum recorded for the 25-residue peptide on the $(M+6H)^{+6}$ ion at m/z 517.95 (MW 3101.65 Da). Observed singly, doubly and triply charged c (red) and z (blue) ions are indicated on the peptide sequence and are labeled in the spectrum. (Ions present in the spectrum are underlined).

doi:10.1371/journal.pone.0117394.g003

and cells were separated and a broad-spectrum protease inhibitor cocktail was added in order to prevent proteolysis of the released peptides. Particle harvesting was then performed from 100 μL of stimulated plasma. The particles were collected and washed, with the captured peptides remaining trapped in the interior of the microparticles. The harvested peptides were eluted and de-salted for mass spectrometry.

The second step in the bioprospecting process is the identification and sequencing of potential CAMPs. The sequences of captured native intact peptides, including potential CAMPs, were elucidated using an Orbitrap Elite mass spectrometer equipped with ETD fragmentation, which has been shown to be ideally suited for fragmenting large, highly charged peptides [14, 15]. When combined with the high sensitivity, resolution and mass accuracy of the Orbitrap, ETD can be used for the *de novo* sequencing of full-length functional peptides. Here, ETD spectra were analyzed by PEAKS software to sequence peptides in a *de novo* manner. PEAKS then uses sequence tags from the *de novo* sequences to search both the American alligator EST database and transcriptome database [16]. However, not all of the *de novo* peptide sequences are found in this database. Peptides of interest, both those that are confirmed from the database and those that have no database equivalent, were all manually verified. To illustrate how peptide sequences can be derived *de novo* from ETD mass spectra, a representative spectrum is presented in Fig. 3.

The bioprospecting CAMP-discovery process, coupling hydrogel particle harvesting of peptides with advanced mass spectrometry techniques, was applied to 100 μL of stimulated alligator plasma. This resulted in the capture of thousands of peptides, all less than 15 kDa in weight, with tandem MS/MS analysis by ETD leading to the sequencing of 568 peptides. A combination of web-based CAMP prediction algorithms and rational peptide sequence analysis based on known CAMP physico-chemical properties are used to identify probable CAMP sequences for synthesis and testing.

Several bioinformatic tools have been developed in an attempt to predict novel CAMPs [10–12]. All 568 sequences were initially submitted for evaluation using three web-based CAMP prediction tools: *CAMP* database, *AntiBP2* and *APD2*. *CAMP* database uses three different algorithms, Support Vector Machine (SVM), Random Forest (RF) and Discriminate Analysis (DA), to predict likelihood of antimicrobial activity [10]. *AntiBP2* uses SVM to determine CAMP potential [11]. The third program, *APD2*, makes a qualitative determination based on the probability of the input sequence being antimicrobial in comparison to the sequences of known antimicrobial peptides in its database [12]. The rational analysis approach focuses on physico-chemical properties that can be calculated based on the peptide sequences, such as molecular weight, length, charge at physiological pH, peptide isoelectric point, and hydrophobicity. Only sequences corresponding to peptides with molecular weights of less than 5.5 kDa were considered, because this is consistent with the molecular weights of the majority of known vertebrate CAMPs. Sequences with charge of +4 or higher were considered, because high cationic character is believed to be linked with activity. Since peptide isoelectric point and hydrophobicity varies widely amongst known CAMPs, these properties were not used as a primary consideration in determining antimicrobial potential. These two approaches to CAMP identification (CAMP-prediction algorithms and rational analysis) are not mutually exclusive, and were both used in order to capture the greatest number of credible peptide sequences that have the potential for antimicrobial activity. Peptide sequences that showed positive CAMP prediction scores or exhibited physico-chemical properties associated with known CAMPs were added to our list of potential CAMPs. The lists of potential CAMPs generated by the two prediction approaches were consolidated and duplicates eliminated.

Based on these prediction methods, forty-five of the 568 peptide sequences were identified as potential CAMPs. From this list, eight peptides ($APOC1_{64-88}$, $APOC1_{67-88}$, $A1P_{394-428}$, $FGG_{398-413}$, $FGG_{401-413}$, AVTG2LP, ASAP130LP and $NOTS_{17-38}$) were selected for further study and synthesized in order to evaluate their antimicrobial activities (Table 1). The peptides $APOC1_{64-88}$, $APOC1_{67-88}$, $A1P_{394-428}$, and ASAP130LP were selected because of their relatively high theoretical positive charges at physiological pH (ranging from +4 to +5), while AVTG2LP and $NOTS_{17-38}$ were selected because the prediction algorithms generally (4 out of 5) agreed that they were likely CAMPs. The two peptides, $FGG_{398-413}$ and $FGG_{401-413}$, were selected due to the overlap exhibited between both prediction methods.

The third step in the CAMP-discovery process is the evaluation of the antimicrobial effectiveness of the newly identified peptides. The eight synthetic peptides were tested against a panel of Gram-positive and Gram-negative bacteria. These bacteria include *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*. Antimicrobial assays designed to determine the half-maximal effective concentrations (EC_{50}) of the peptides were performed using a high-throughput assay based on resazurin as a reporter for cell viability (Table 2) [17–24]. When incubated with live metabolically-active cells, the non-fluorescent blue dye resazurin is converted to the highly fluorescent pink resorufin, as a result of reduction by the cells. While bacteria can reduce resazurin via multiple metabolic processes, the rate of conversion and the corresponding increase in fluorescence are directly proportional to the number of living cells in the sample [19–21, 25]. Thus, fluorometric detection of the rate of

Table 1. Sequences and Physico-chemical Properties of Novel Alligator CAMPs.

Peptide	Sequence	Length (res)	Molecular Weight (Da)	Net Charge	pI	Hydrophobicity	Parent Protein
$APOC1_{64–88}$	FSTKTRNWFSEHFKKVKEKLKDTFA	25	3101.65	4	10.0	-1.2	Apolipoprotien C1
$APOC1_{67–88}$	KTRNWFSEHFKKVKEKLKDTFA	22	2766.49	4	10.0	-1.4	Apolipoprotien C1
$FGG_{398–413}$	YSLKKTSMKIIPFTRL	16	1925.12	4	10.5	-0.050	Fibrinogin
$FGG_{401–413}$	KKTSMKIIPFTRL	13	1561.94	4	11.3	-0.19	Fibrinogin
$A1P_{394–428}$	PPPVIKFNRPFLMWIVERDTRSILFMGKIVNPKAP	35	4106.28	4	11.0	0.020	Alpha-1-antiproteinase
AVTG2LP*	LQTKLKKLLGLESVF	15	1716.06	2	9.70	0.36	Vitellogenin-2
ASAP130LP*	PPGASPRKKPRKQ	13	1445.85	5	12.0	-2.3	Sin3A-Associated Protein, 130 kDa
$NOTS_{17–38}$	VERIPLVRFKSIKKQLHERGDL	22	2660.56	3	10.3	-0.62	Nothepsin

The physico-chemical properties for eight novel alligator CAMPs identified via the process. The peptide name is determined based on the parent protein with the amino acid sequence numbers in the subscript.

* These peptides were *de novo* identified.

doi:10.1371/journal.pone.0117394.t001

resazurin conversion to resorufin at $530_{ex}/590_{em}$ allows for quantification of bacterial survival following exposure to antibacterial compounds, such as CAMPs [17]. It has been confirmed that the time that bacterial cultures require to achieve specified fluorescence intensities correlates inversely to the initial bacterial concentration, and the results obtained using resazurin-based assays are comparable to those determined using classical dilution-plating assays for evaluating bacterial viability [17, 18, 23, 24].

Of the eight synthesized peptides, five showed significant antibacterial activity against one or more of the bacteria in the panel, based on EC_{50} values (Fig. 4): $APOC1_{64–88}$, $APOC1_{67–88}$, $A1P_{394–428}$, $FGG_{398–413}$ and $FGG_{401–413}$. Based on CAMP prediction algorithms, $NOTS_{17–38}$ and AVTG2LP were predicted likely to be effective antimicrobial peptides (Table 3). However, neither of these peptides exhibited significant antibacterial activity against the panel of bacteria (Table 2), revealing limitations in the utility of currently available CAMP prediction models [10–12]. Additionally, the peptide ASAP130LP, which appeared to have physico-chemical properties consistent with known CAMPs, also proved ineffective against the panel of bacteria tested.

$APOC1_{64–88}$ (25aa) and $APOC1_{67–88}$ (22aa) are a nested pair of peptide fragments of apolipoprotein C1 and share a nominal net charge of +4 at physiological pH. Apolipoprotein C1 is generally associated with lipid metabolism, but it has recently been suggested that the protein may also play a role in inflammation [26]. $APOC1_{64–88}$ and $APOC1_{67–88}$ exerted significant antimicrobial activity against *E. coli*, *P. aeruginosa* and *B. cereus*, but were not as effective against *S. aureus*. The longer peptide $APOC1_{64–88}$ exhibited antimicrobial EC_{50} values of 0.192 μM and 1.41 μM against the Gram-negative bacteria *E. coli* and *P. aeruginosa*, respectively, and 0.245 μM and 9.66 μM against the Gram-positive bacteria *B. cereus* and *S. aureus*, respectively. $APOC1_{64–88}$ showed substantial activity against *E. coli, P. aeruginosa* and *B. cereus* when compared to the performance of the known CAMP LL-37, which had EC_{50} values of 0.00821 μM, 0.525 μM and 0.0287 μM, against these bacteria, respectively. The nested peptide $APOC1_{67–88}$ exhibited increased potency, relative to the longer peptide, with EC_{50} values of 0.151 μM, 0.948 μM, 0.210 μM and 7.08 μM against *E. coli, P. aeruginosa B. cereus*, and *S. aureus*, respectively. The performance of $APOC1_{67–88}$ was similar to EC_{50} values obtained by LL-37. Interestingly, neither APOC1 peptide was predicted to be strongly antimicrobial by the CAMP prediction algorithms tested, again highlighting the difficulty of predicting antimicrobial

Table 2. Antibacterial Performance Data for Alligator CAMPs.

Peptide	*E. coli*		*B. cereus*		*P. aeruginosa*		*S. aureus*	
	EC_{50} (μM)	95% CI	EC_{50} (μM)	95% CI	EC_{50} (μM)	95% CI	EC_{50} (μM)	95% CI
LL-37	0.00821	0.00591 to 0.0113	0.0287	0.0242 to 0.0341	0.525	0.446 to 0.615	0.552	0.383 to 0.797
$APOC1_{64–88}$	0.192	0.129 to 0.284	0.245	0.223 to 0.269	1.41	0.906 to 2.23	9.66	7.69 to 12.2
$APOC1_{67–88}$	0.151	0.0716 to 0.319	0.210	0.181 to 0.244	0.948	0.706 to 1.27	7.08	5.39 to 9.29
$FGG_{398–413}$	0.332	0.162 to 0.678	9.35	7.75 to 11.3	7.02	5.30 to 9.23	2.84	1.86 to 4.33
$FGG_{401–413}$	0.245	0.150 to 0.360	18.7	wide	11.1	9.30 to 13.4	31.8	18.7 to 54.0
$A1P_{394–428}$	0.0986	0.0478 to 0.203	0.770	0.257 to 2.31	4.35	3.74 to 5.04	1.36	0.925 to 1.99
AVTG2LP	NA	NA	NA	NA	NA	NA	NA	NA
ASAP130LP	NA	NA	NA	NA	NA	NA	101	wide
$NOTS_{17–38}$	NA	NA	NA	NA	NA	NA	198	wide

Antibacterial activities against *E. coli, B. cereus, P. aeruginosa* and *S. aureus* are expressed in terms of EC_{50} (μM) values with corresponding 95% confidence interval (CI) range. The human CAMP LL-37 is used as a standard for assessing antibacterial performance [30, 31]. **NA** = no significant activity at the highest peptide concentration tested against the listed bacteria.

doi:10.1371/journal.pone.0117394.t002

peptides based on our current understanding of the factors defining peptide antimicrobial activity. Only the APD2 database correctly predicted that these peptides would have antimicrobial activity.

$A1P_{394–428}$, a fragment of alpha-1-antiproteinase, is a 35-residue peptide with a predicted +4 charge at neutral pH. Alpha-1-antiproteinase, also known as alpha-1-antitrypsin, is a serine protease inhibitor (Serpin), and is a major protease inhibitor present in human body that has been linked to anti-inflammatory immune response [27]. Although 4/5 of the CAMP-predictor algorithms indicated it would not be antimicrobial, the peptide showed significant activity against *E. coli* (0.0986 μM), *B. cereus* (0.770 μM) and *S. aureus* (1.36 μM), and exhibited moderate activity against *P. aeruginosa* (4.35 μM). Based on its performance relative to that of LL-37 against the panel of bacteria, $A1P_{394–428}$ appears to show good broad-spectrum antimicrobial effectiveness. Only the APD2 database correctly predicted that this peptide would have antimicrobial activity.

Two nested peptides derived from fibrinogen, $FGG_{398–413}$ (16aa) and $FGG_{401–413}$ (11aa) were generated, both of which carry a nominal charge of +4 at physiological pH. Fibrinogen has been associated with coagulation, which is initiated through proteolytic processing via thrombin. However, it has recently been suggested that treatment of fibrinogen with thrombin may also generate peptides that exhibit antimicrobial activity [28]. $FGG_{401–413}$ was predicted by all but one of the algorithms to have antimicrobial activity, while only 2 out of 5 algorithms tested predicted $FGG_{398–413}$ to be a CAMP. Interestingly, neither $FGG_{398–413}$ nor $FGG_{401–413}$ was found to have strong antimicrobial activity except against *E. coli* (EC_{50} = 0.33 μM and 0.245 μM, respectively). However, $FGG_{398–413}$ proved to be moderately effective against Gram-positive *S. aureus* (EC_{50} = 2.84 μM). Against *P. aeruginosa* and *B. cereus*, $FGG_{398–413}$ exhibited poor antimicrobial activity with EC_{50} values of 7.02 μM and 9.35 μM, respectively. The nested peptide $FGG_{401–413}$ displayed a decrease in potency relative to $FGG_{398–413}$ against *P. aeruginosa*, *B. cereus*, and *S. aureus* with EC_{50} values of 11.1 μM, 18.7 μM and 31.8 μM, respectively. While $FGG_{398–413}$ presented mixed antimicrobial effectiveness when compared to the performance of LL-37, the nested peptide $FGG_{401–413}$ showed fairly poor antimicrobial activity against the bacteria tested, except for *E. coli*. These FGG peptides potentially represent a counter-example to the APOC1 peptides, which were active but were predicted not to be, except for the APD2 database. In this case, only APD2 correctly predicted that the FGG peptides would be inactive.

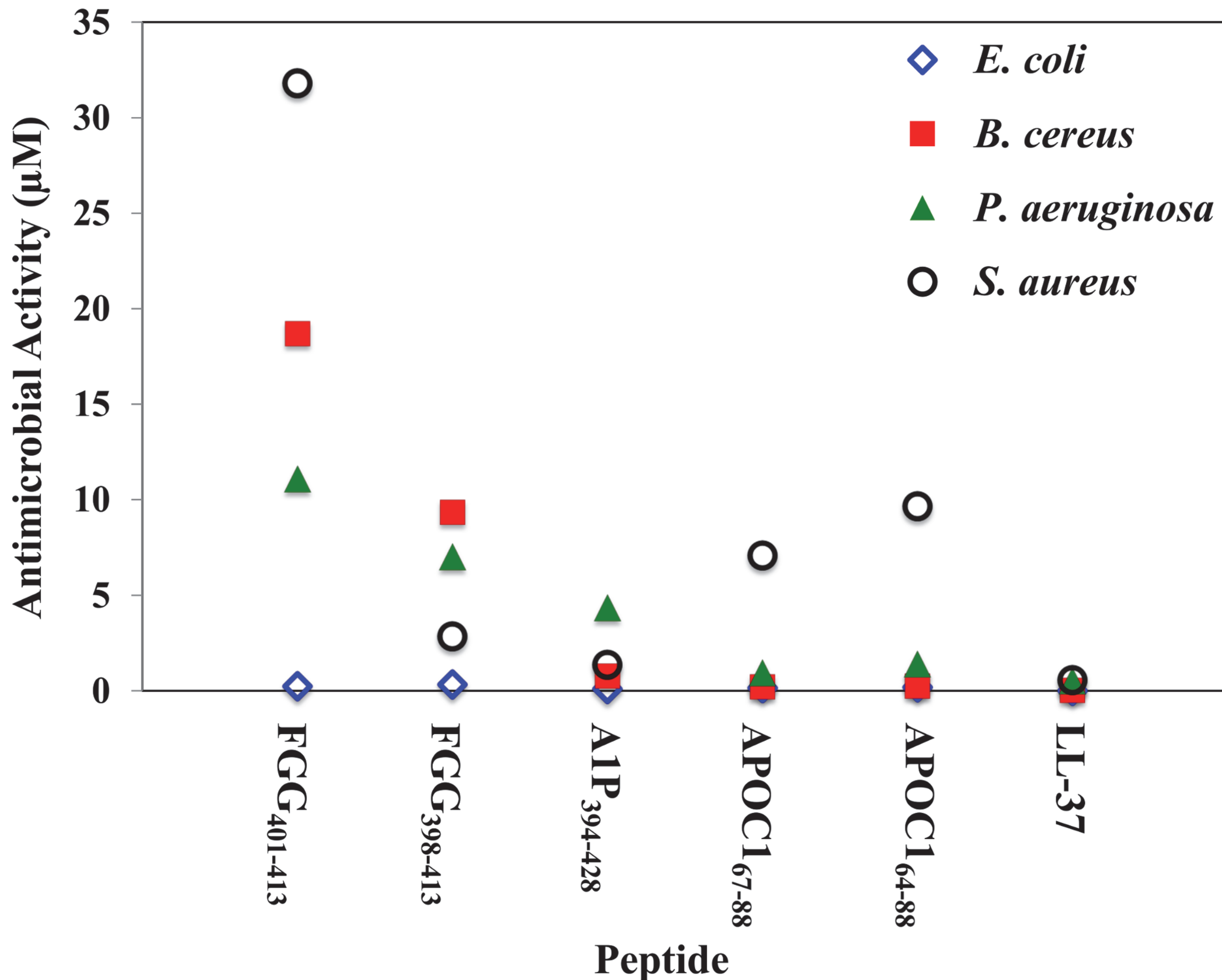

Fig 4. Potencies of LL-37 and Five Novel Alligator CAMPs. Comparison of the antibacterial effectiveness of each peptide against *E. coli, B. cereus, P. aeruginosa* and *S. aureus*, expressed in terms of EC_{50} (μM) values. Bacterial survival results generated for each CAMP are fit to a variable-slope sigmoidal regression model to reveal bacterial survival curves to ascertain EC_{50} values.

doi:10.1371/journal.pone.0117394.g004

Based on the antimicrobial performance data for the eight selected peptides, *E. coli* (ATCC 25922) appears to be very sensitive to CAMP activity (Fig. 4), suggesting that performance against this strain may not be a good predictor of broad-spectrum activity. This is reflected in the performance of the peptides against the three other bacteria tested, emphasizing the importance of testing peptides against multiple strains and Gram-types of bacteria for a more complete view of their potential activity.

Discussion

In order to overcome the limitations associated with current approaches to CAMP discovery, we have developed a novel and promising method for identifying new and potentially useful antimicrobial peptides. In this process, hydrogel microparticles harvest functional CAMPs based on their physico-chemical properties. Coupled with subsequent mass spectral analysis of the intact captured peptides, this process eliminates current labor-intensive, low-yield

Table 3. CAMP prediction results.

Peptide	*CAMP* Prediction Score			*AnitBP2* Prediction Score SVM	*APD2* Prediction Probability Qualitative
	SVM	RF	DA		
$APOC1_{64–88}$	0.894:Non-AMP	0.728:Non-AMP	0.667:Non-AMP	-0.210:Non-AMP	+
$APOC1_{67–88}$	0.598:Non-AMP	0.692:Non-AMP	0.352:Non-AMP	-0.052:Non-AMP	+
$FGG_{398–413}$	0.508:AMP	0.656:Non-AMP	-0.384:AMP	-0.172:Non-AMP	-
$FGG_{401–413}$	0.732:AMP	0.514:AMP	-1.23:AMP	ND*	-
$A1P_{394–428}$	0.935:Non-AMP	0.838:Non-AMP	0.363:Non-AMP	-0.241:Non-AMP	+
AVTG2LP	0.821:AMP	0.386:Non-AMP	0.877:AMP	0.223:AMP	+
ASAP130LP	0.157:Non-AMP	0.445:Non-AMP	0.077:Non-AMP	ND*	+
$NOTS_{17–38}$	0.757:AMP	0.600:AMP	-0.165:Non-AMP	0.618:AMP	+

Using 3 different web-based CAMP prediction applications (*CAMP database, AntiBP2 and APD2*) each peptide was scored and given a prediction of whether it would have antimicrobial activity (AMP) or not (Non-AMP).

* *AntiBP2* requires the sequence length of $\geq$ 15 amino acids for a prediction score.

CAMP database: http://www.camp.bicnirrh.res.in/.

AntiBP2: http://www.imtech.res.in/raghava/antibp2/.

APD2: http://aps.unmc.edu/AP/prediction/prediction_main.php.

doi:10.1371/journal.pone.0117394.t003

processes associated with conventional approaches for CAMP identification. As an initial proof-of-principle, we demonstrated the ability of the hydrogel particles to harvest known CAMPs from plasma using commercial alligator plasma spiked with buforin, SMAP-29, and indolicidin. The particles were found to capture the model CAMPs from plasma and subsequently release them for analysis by mass spectrometry.

Application of the particle-based CAMP discovery process to a 100 μL sample of plasma from the American alligator resulted in the capture and sequencing of 568 alligator peptides. Evaluation of these sequences using a combination of rational analysis and web-based predictor algorithms identified 45 potential CAMPs. Eight of these peptides were synthesized and their antimicrobial effectiveness evaluated, which resulted in the discovery of five peptides ($APOC1_{64–88}$, $APOC1_{67–88}$, $A1P_{394–428}$, $FGG_{398–413}$ and $FGG_{401–413}$) that exhibited antimicrobial activity against one or more of the Gram-positive and/or Gram-negative bacteria tested.

We have identified at least one peptide ($APOC1_{67–88}$) with significant antimicrobial activity against the Gram-negative bacterium *P. aeruginosa* (EC_{50} = 0.948 μM), similar to the activity of the well-studied CAMP LL-37 (EC_{50} = 0.525 μM). This peptide is also able to achieve 50% killing of *B. cereus* cells at a concentration within an order of magnitude of the LL-37 EC_{50} value. We also identified $A1P_{394–428}$ as a peptide that has significant activity against the Gram-positive bacterium *S. aureus* (EC_{50} = 1.36 μM), which is similar to that of LL-37 (EC_{50} = 0.552 μM).

The sequences of these new peptides are not derived from any of the known classes of CAMPs, but instead are fragments of larger proteins with diverse functions. The *in vivo* role of these peptides or their source proteins in innate immunity or host defense of the American alligator is not yet known, and will be the subject of further study.

The successful identification of five novel peptides that exhibit the ability to exert a direct antimicrobial effect on bacteria from 100 μL of alligator plasma substantiates the potential utility of this new CAMP-discovery process. However, the mixed performance of the prediction algorithms and databases to accurately predict the activity of the eight selected peptides exposes limitations associated the existing algorithms, and more broadly our understanding of the relationship between peptide physico-chemical properties and CAMP effectiveness. These results

suggest that the data upon which the prediction algorithms [10–12] are based fail to sufficiently capture the sequence and performance diversity that exists among antimicrobial peptides.

Combined, the data from the *P. aeruginosa*, *S. aureus* and *B. cereus* bacterial assays suggest that three of the new alligator peptides have significant and potentially broad-spectrum antibacterial activity ($APOC1_{64-88}$, $APOC1_{67-88}$, $A1P_{394-428}$), while two do not ($FGG_{398-413}$ and $FGG_{401-413}$), when compared to the human peptide LL-37. In performance assays, *E. coli* was affected by all 5 peptides with roughly the same sensitivity, suggesting that this strain is very sensitive to CAMPs, and is less able to discriminate between peptides and the scope of their effectiveness. Therefore, the following analysis focuses on their performance against *P. aeruginosa*, *S. aureus* and *B. cereus*. Calculations from the *CAMP* program [10] did not accurately predict the positive antimicrobial activity of any of the active peptides in this study. The *CAMP* program's SVM calculation was correct in predicting the non-activity of one peptide (ASAP130LP), while *CAMP* program's DA calculation only correctly predicted the non-activity of two peptides (ASAP130LP and $NOTS_{17-38}$). *AntiBP2*'s prediction of activity was incorrect for each of the 6 peptides for which values were computed. The *APD2* database [12] was perhaps the most accurate, correctly predicting the activity for 5/8 peptides, and was the only database to positively predict the activity of the three active peptides ($APOC1_{64-88}$, $APOC1_{67-88}$, $A1P_{394-428}$), and the inactivity of the two FGG peptides. This result may reflect the better potential predictive power of this database, built upon homology of the query peptide against a large collection of verified and active peptides from the literature. However, the *APD2* database incorrectly predicted that 3 of the inactive peptides would be active (AVTG2LP, ASAP130LP, $NOTS_{17-38}$).

This first application of the bioprospecting approach to CAMP discovery has yielded immediate success in the form of newly identified peptides; however, the greatest benefit may lie in the insights that it has provided into how to improve the process. Enhancing CAMP harvesting selectivity and modifying chromatography and mass spectrometry parameters will allow more efficient capture and identification of CAMP candidates. Furthermore, until more reliable algorithms become available, it will be necessary to continue casting a relatively wide net when predicting whether captured peptides are potential CAMPs. We anticipate that these efforts will not only lead to the discovery of new CAMPs, but will generate a body of sequence and performance data for both CAMPs and CAMP-like peptides that can be used to develop new or refine existing CAMP-prediction algorithms in order to improve their reliability and versatility. Such developments would in turn improve the efficiency and utility of the bioprospecting CAMP discovery process.

Although this CAMP discovery process has only been used to analyze samples of alligator plasma to date, the relatively small sample volume requirement and the fact that the process is sample agnostic make it applicable to a broad spectrum of animals that were previously thought inaccessible, such as organisms of smaller body mass or endangered species. This will allow analysis of the peptidomes in some of the world's most remarkable species, to dramatically expand the current CAMP library and potentially unlock strategies for overcoming antibiotic resistance via the discovery of new antimicrobial peptides. Beyond CAMP discovery, we envision the bioprospecting approach being applied to mining peptidomes for other classes of peptides for therapeutic and biotechnology applications.

Materials and Methods

Bacterial Strains

Escherichia coli (ATCC 25922), *Bacillus cereus* (ATCC 11778), *Pseudomonas aeruginosa* (ATCC 9027), and *Staphylococcus aureus* (ATCC 25923) used in these studies were purchased from the American Type Culture Collection (Manassas, VA). Bacteria were grown following

ATCC recommended protocols for each strain and frozen aliquots were prepared in 20% glycerol and stored at -80°C for further use.

Materials

The peptides used in these studies were custom synthesized by ChinaPeptides Company (Shanghai, China) and had purities of $\geq$ 95%, based on chromatographic analysis of the purified peptides. Synthetic peptides were verified on a Thermo LTQ mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The broad-spectrum protease inhibitor cocktail Protease cOmplete was purchased from Roche Diagnostic, Corp. (Indianapolis, IN). Resazurin, sodium salt is purchased from Sigma-Aldrich (St. Louis, MO). *N*-Isopropylacrylamide (NIPAm), *N*, *N*′-Methylenebisacrylamide (BIS), Acrylic acid (AAc), 2-Acrylamido-2-methylpropane sulfonic acid (AMPS), Methyl Acrylate (MA), Lithium hydroxide (LiOH) and potassium persulfate (KPS) are all purchased from Sigma-Aldrich (St. Louis, MO). Mueller Hinton Broth (MHB) was purchased from Becton Dickinson and Company (Sparks, MD). Phosphate buffered saline (PBS) was purchased from Corning-cellgro (Manassas, VA). Commercial alligator plasma was purchased from Bioreclamation (Westburg, NY). Alligator blood was acquired from St. Augustine's Alligator Farm (St. Augustine, FL). All experiments involving alligators were carried out with compliance with relevant guidelines, using protocols approved by the GMU IACUC.

Particle Synthesis

The p-NIPAm-based particles are synthesized using one-pot free radical precipitation polymerization following previously published protocols [8]. Particles incorporating AAc and AMPS are synthesized as follows: NIPAm (2.98 g, 26.28 mmol), BIS (0.111 g, 0.72 mmol), AAc (370 μL, 5.4 mmol), and AMPS (0.746 g, 3.69 mmol) are dissolved in 120 mL H_2O. The reaction is heated to 72–78°C with stirring while degassing with N_2. Once the reaction has stabilized at 77°C, the polymerization is initiated with the addition of KPS (24 mg, 8.88 μmol), and allowed to continue for three hours at 77°C under N_2. The reaction is allowed to cool and the resulting particle suspension is dialyzed against water at room temperature for three days, with the dialyzed particles lyophilized and ready for use in harvesting. Core-shell particles incorporating AAc are synthesized using a similar approach, with NIPAm (1.08 g, 9.54 mmol), BIS (55.5 mg, 0.36 mmol), and MA (734 μL, 8.10 mmol) as the initial monomer feed dissolved in 60 mL H_2O. The shell is introduced three hours after initiation, with the addition of a new combination of feed monomers, NIPAm (2.0 g, 17.64 mmol) and BIS (55.5 mg, 0.36 mmol) in 60 mL H_2O. The reaction is allowed to continue with stirring for another 3 hours under N_2 at 74°C. Particles are dialyzed to remove unreacted monomer and byproducts. The core-shell MA particles are saponified using lithium hydroxide in aqueous methanol to convert the MA units to AAc. The hydrated diameters of the particles are determined using dynamic light scattering at a scattering angle of 90°. The AAc/AMPS particles were determined to be 591.9 ± 78.6 nm in diameter and the core-shell AAc particles 1290 ± 214 nm. The particles are combined in a 50:50 mixture by weight for use in harvesting.

Harvest and Elution

Alligator plasma* (100 μL) is diluted into 1.6 mL of Hydrogel particles (40 mg) suspended in aqueous 10 mM Tris-Cl for a final volume of ~1.7 mL and pH of 5. After incubating approximately 18 hours at room temperature, the plasma–particle harvest mixture is centrifuged at 16.1×10^3 *rcf* to pellet the particles, and the pelleted particles are resuspended in 10 mM Tris-Cl buffer (pH 7.4). This centrifugation and resuspension process is repeated at least two times

to ensure removal of excluded proteins and peptides. Following the final wash with Tris-Cl buffer, the pelleted particles are suspended in an elution solution of 1:1 trifluoroethanol (TFE): 0.1% TFA in water. The particles are gently agitated for one hour at room temperature before pelleting (as described above). The supernatant layer, containing eluted captured peptides, is set aside for later use. To ensure all peptides had been removed from the particle interior, the elution process is repeated three more times with 20 minute incubations. All elution supernatants are combined and dried via speed vacuum before de-salting by solid-phase extraction with a C_{18} Zip-Tip (Millipore, Billerica, MA, USA) for mass spectrometry analysis.

* In the case of the model plasma sample, commercial alligator plasma was spiked with the three CAMPs buforin, SMAP-29, and indolicidin (60 pmol each). For CAMP discovery, plasma from ionomycin stimulated alligator blood (1 μM, 30 minutes, 30°C) is used. Immediately following stimulation, an aliquot of protease inhibitor solution (10 μL / 100 μL of plasma), is added to the stimulated plasma.

LC-MS/MS

Particle eluate is analyzed by high-sensitivity nanospray LC–MS/MS with an LTQ-Orbitrap Elite mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an EASY-nLC 1000 HPLC system (Thermo Fisher Scientific, Waltham, MA, USA). The reversed-phase LC column is a PepMap 50 μm i.d. × 15 cm long with 3 μm, 100 Å pore size, C_{18} resin (Thermo Fisher Scientific, Waltham, MA, USA). The mobile phase is a gradient prepared from 0.1% aqueous formic acid (mobile phase component A) and 0.1% formic acid in acetonitrile (mobile phase component B). After sample injection, the column is washed for 5 min with A; the peptides are eluted by using a linear gradient from 0 to 50% B over either 45 min or 2 hours and ramping to 100% B for an additional 2 min; the flow rate is 300 nL/min. The LTQ-Orbitrap Elite is operated in a data-dependent mode in which each full MS scan (120,000 resolving power) is followed by five MS/MS scans (120,000 resolving power) in which the five most abundant molecular ions are dynamically selected and fragmented by electron transfer dissociation (ETD) using fluoranthene as the electron transfer reagent. “FT master scan preview mode”, “Charge state screening”, “Monoisotopic precursor selection”, and “Charge state rejection” were enabled so that only the $\geq$ 3+ ions are selected and fragmented by ETD.

Tandem mass spectra were imported directly as .RAW files and analyzed by PEAKS *de novo* sequencing software version 6 (Bioinformatics Solutions Inc., Waterloo, ON Canada). PEAKS first performs a *de novo* sequence analysis using the ETD MS/MS data. Mass tolerance for precursor ions was 10 ppm and mass tolerance for fragment ions was 0.05 Da. Data were analyzed with no enzyme specificity, along with oxidation (+15.9949 Da) on methionine as a variable post translation modification. Confident *de novo* peptide identifications were achieved by filtering Average Local Confidence (ALC) to $\geq$ 30%. Sequence tags from the confident *de novo* sequences are searched against 2 separate databases. The first is an expressed sequence tag (EST) database obtained by searching the EST database at NCBI (http://www.ncbi.nlm.nih.gov) for all known alligator EST sequences. A total of 5469 alligator EST sequences are found from a number of sources, including the Adult American Alligator Testis Library (University of Florida, Department of Zoology, Gainesville, FL), the Juvenile American Alligator Liver Library (NIBB, Japan), and the Adult American Alligator Liver Library (University of Florida, Department of Zoology, Gainesville, FL). All were downloaded to the local computer hard drive and subsequently uploaded as a database in PEAKS. The second database was an *Alligator mississippiensis* transcriptome obtained from the International Crocodilian Genome Working Group (www.crocgenomes.org) [16]. A 1% false discovery rate (FDR) was used as a cut-off value for reporting peptide spectrum matches (PSM) from either database. Peptides of interest, both those that are

sequenced from the databases and those that have only a *de novo* sequence and thus no database equivalent are all manually verified (Fig. 3). For *de novo*-only sequences, only leucine (L) was denoted since it is indistinguishable from isoleucine (I) by ETD fragmentation.

CAMP prediction

Verified sequences were input into web-based CAMP prediction sites, *CAMP* database [10], *AntiBP2* [11] *and APD2* [12], where each peptide was scored and the likelihood of their having antimicrobial activity predicted (Table 3). Furthermore, the physico-chemical properties (length, molecular weight, nominal solution charge, pI and hydrophobicity) of all verified sequences were calculated and sorted. Length, charge and hydrophobicity were calculated using the *CAMP* database properties calculator [10]. *CAMP* database calculates hydrophobicity based on the per-residue hydrophobicity scale determined by George Rose *et al.* [29]. Molecular weight and pI were calculated using ExPASy compute MW/pI tool (http://web.expasy.org/compute_pi/).

Antibacterial Performance

Frozen enumerated bacterial aliquots were thawed on ice and mixed. For each strain, bacteria are diluted to 2×10^6 CFU/mL in sterile 10 mM sodium phosphate (pH 7.4) and added in 50 μL aliquots to the wells of a 96-well black microtiter plate (Greiner Bio-One 655201) containing 50 μL volumes of serially diluted CAMP, dissolved in the same phosphate buffer. Control wells contain bacteria with no peptide. The microtiter plate was incubated for 3 hours at 30°C (*B. cereus*) or 37°C for other strains. After three hours, 100 μL of PBS solution with dissolved resazurin and MHB was added to each well. The amount of resazurin and MHB that was added is bacterial strain dependent, with the final resazurin (μM)/ MHB (wt/vol) concentrations being 100 μM/ 0.2% (wt/vol) for *E. coli*, 12.5 μM/ 0.05% (wt/vol) for *B. cereus*, 25 μM/ 2.2% (wt/vol) for *P. aerugoinsa*, and 50 μM/ 2.2% (wt/vol) for *S. aureus*. The metabolic conversion of resazurin to resorufin is bacterial strain dependent and requires concentrations optimized for each species to achieve a high-throughput assessment. Following addition of resazurin/MHB buffer, the plate was immediately placed in either a SpectraMax Gemini EM plate-reading fluorimeter (*E. coli* and *B. cereus*) or a TeCan Safire 2 fluorimeter (*S. aureus* and *P. aeruginosa*) for incubation overnight at either 30°C (*B. cereus*) or 37°C (other strains) while monitoring fluorescence for each well.

Fluorescence data was collected from each well during the monitoring period using equations compiled by microplate data software (SoftMax Pro 4.5 or Magellen 6). Onset time of half maximal fluorescence ($T_{0.5}$) was used for quantifying bacterial concentrations. Standard curves were generated in preliminary experiments using serially diluted bacterial suspensions (~10^6 CFU/mL- 10^3 CFU/mL) without CAMPs. Observed $T_{0.5}$ values are plotted against initial CFU counts that had been determined by plating on MHB agar plates, and the relationships analyzed by linear regression, affording the following equations:

$$\log(\mathrm{CFU}_{E.coli}) = (-1.94 \times 10^{-4})(T_{0.5}) + 8.28(R^2 = 0.999) \tag{1}$$

$$\log(\mathrm{CFU}_{B.cereus}) = (-1.42 \times 10^{-4})(\mathrm{T}_{0.5}) + 5.30(\mathrm{R}^2 = 0.995) \tag{2}$$

$$\log(\mathrm{CFU}_{P.aeruginosa}) = (-1.25 \times 10^{-4})(\mathrm{T}_{0.5}) + 9.22(\mathrm{R}^2 = 0.991) \tag{3}$$

$$\log(\mathrm{CFU}_{S.aureus}) = (-9.03 \times 10^{-4})(\mathrm{T}_{0.5}) + 7.99(\mathrm{R}^2 = 0.993) \tag{4}$$

These linear regression equations are used to interpolate survival following incubation of bacteria with CAMPs, with the CFU for each well being determined based on their respective $T_{0.5}$ values. Correlating bacterial CFU values for wells containing peptide with control wells containing no CAMPs it is possible to determine bacterial survival for wells containing CAMPs. The resazurin assay was validated with traditional dilution plating assays and the resazurin-based values were found not significantly different based on t-test and F-test statistical evaluations (see Table A and Fig. A in S1 File).

Statistical Analysis

Antibacterial measurements are performed in triplicate. Bacterial survival results generated for each CAMP are fit to a variable-slope sigmoidal regression model to reveal bacterial survival curves using Prism 5 (GraphPad Software, Inc). Best-fit values generated for the survival curve-fit parameter log (EC_{50}) are used as performance criteria. Log (EC_{50}) represents the log of the peptide concentration (PC) that causes a halfway response between S_{min} and S_{max}, the minimal and maximal survival values, respectively, where Hill slope (HS) is the parameter used to quantify the steepness of the transition slopes in sigmoidal survival curves.

$$\text{Bacterial Survival} = S_{min} + \frac{S_{max} - S_{min}}{(1 + 10^{((\log(EC_{50})) - ((\log(PC)) * HS))})} \quad (5)$$

Antilogs of the log (EC_{50}) values, the EC_{50} values, are tabulated and 95% confidence intervals (CI) are presented to demonstrate overlap and statistical significance. This data is presented in Table 2 and in graphical format in Fig. 4.

Supporting Information

S1 File. This file contains Table A and Figure A. Table A, Comparison of Antibacterial Performance EC_{50} and Hill Slope Values Determined by Dilution Plating and by Resazurin Assays. EC_{50} and Hill slope values for LL-37 against *E. coli* and *B. cereus* with corresponding 95% confidence interval range. Figure A, Comparison of Antibacterial Performance Determined by Dilution Plating and Resazurin Assays. Antibacterial effectiveness of LL-37 against *E. coli* (A) and *B. cereus* (B) comparing results from dilution plating (represented in open circles = ○) and resazurin assays (validation data represented in open squares = □; reported manuscript data represented in closed triangles = ▲). Data were fit to equation 5, a standard variable slope dose-response equation, in order to obtain EC_{50} and Hill slope values.
(DOCX)

Acknowledgments

We gratefully acknowledge the St. Augustine Alligator Farm Zoological Park and its staff for providing alligator blood samples and the International Crocodilian Genome Working Group (www.crocgenomes.org) for access to the *Alligator mississippiensis* transcriptome. We would also like to thank Dr. Jennifer Van Eyk (Johns Hopkins University) for use of their Thermo Scientific Orbitrap Elite and helpful discussions.

Author Contributions

Conceived and designed the experiments: BMB MVH. Performed the experiments: MLJ MCD SMB CAR MCC PSR. Analyzed the data: MLJ MCD SMB CAR MCC PSR. Contributed reagents/materials/analysis tools: KAV. Wrote the paper: BMB MVH MLJ JMS. Advised on reptiles and reptile biology: KAV.

References

1. Hancock REW, Sahl H-G (2006) Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. Nat Biotechnol 24: 1551–1557. doi: 10.1038/nbt1267 PMID: 17160061
2. Zasloff M (2002) Antimicrobial peptides of multicellular organisms. Nature 415: 389–395. doi: 10.1038/415389a PMID: 11807545
3. Merchant ME, Leger N, Jerkins E, Mills K, Pallansch MB, et al. (2006) Broad spectrum antimicrobial activity of leukocyte extracts from the American alligator (Alligator mississippiensis). Vet Immunol Immunopathol 110: 221–228. doi: 10.1016/j.vetimm.2005.10.001 PMID: 16298430
4. Merchant ME, Roche C, Elsey RM, Prudhomme J (2003) Antibacterial properties of serum from the American alligator (Alligator mississippiensis). Comp Biochem Physiol Part B Biochem Mol Biol 136: 505–513. doi: 10.1016/S1096-4959(03)00256-2
5. Darville LNF, Merchant ME, Hasan A, Murray KK (2010) Proteome analysis of the leukocytes from the American alligator (Alligator mississippiensis) using mass spectrometry. Comp Biochem Physiol Part D Genomics Proteomics 5: 308–316. doi: 10.1016/j.cbd.2010.09.001 PMID: 20920849
6. Van Hoek ML (2014) Antimicrobial peptides in reptiles. Pharmaceuticals (Basel) 7: 723–753. Available: http://www.ncbi.nlm.nih.gov/pubmed/24918867. Accessed 2014 Jun 16. doi: 10.3390/ph7060723
7. Pata S, Yaraksa N, Daduang S, Temsiripong Y, Svasti J, et al. (2011) Characterization of the novel antibacterial peptide Leucrocin from crocodile (Crocodylus siamensis) white blood cell extracts. Dev Comp Immunol 35: 545–553. doi: 10.1016/j.dci.2010.12.011 PMID: 21184776
8. Luchini A, Geho DH, Bishop B, Tran D, Xia C, et al. (2008) Smart hydrogel particles: biomarker harvesting: one-step affinity purification, size exclusion, and protection against degradation. Nano Lett 8: 350–361. doi: 10.1021/nl072174l PMID: 18076201
9. Longo C, Patanarut A, George T, Bishop B, Zhou W, et al. (2009) Core-shell hydrogel particles harvest, concentrate and preserve labile low abundance biomarkers. PLoS One 4: e4763. doi: 10.1371/journal.pone.0004763 PMID: 19274087
10. Thomas S, Karnik S, Barai RS, Jayaraman VK, Idicula-Thomas S (2010) CAMP: a useful resource for research on antimicrobial peptides. Nucleic Acids Res 38: D774–80. Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2808926&tool = pmcentrez&rendertype = abstract. Accessed 2014 Feb 9. doi: 10.1093/nar/gkp1021 PMID: 19923233
11. Lata S, Sharma B, Raghava G (2007) Analysis and prediction of antibacterial peptides. BMC Bioinformatics 10: 1–10. Available: http://www.biomedcentral.com/1471-2105/8/263. Accessed 2014 Feb 10.
12. Wang G, Li X, Wang Z (2009) APD2: the updated antimicrobial peptide database and its application in peptide design. Nucleic Acids Res 37: D933–D937. doi: 10.1093/nar/gkn823 PMID: 18957441
13. Sørensen O, Arnljots K, Cowland JB, Bainton DF, Borregaard N (1997) The human antibacterial cathelicidin, hCAP-18, is synthesized in myelocytes and metamyelocytes and localized to specific granules in neutrophils. Blood 90: 2796–2803. PMID: 9326247
14. Syka JEP, Coon JJ, Schroeder MJ, Shabanowitz J, Hunt DF (2004) Peptide and protein sequence analysis by electron transfer dissociation mass spectrometry. Proc Natl Acad Sci U S A 101: 9528–9533. doi: 10.1073/pnas.0402700101 PMID: 15210983
15. Mikesh LM, Ueberheide B, Chi A, Coon JJ, Syka JEP, et al. (2006) The utility of ETD mass spectrometry in proteomic analysis. Biochim Biophys Acta 1764: 1811–1822. doi: 10.1016/j.bbapap.2006.10.003 PMID: 17118725
16. St John JA, Braun EL, Isberg SR, Miles LG, Chong AY, et al. (2012) Sequencing three crocodilian genomes to illuminate the evolution of archosaurs and amniotes. Genome Biol 13: 415. Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3334581&tool = pmcentrez&rendertype = abstract. doi: 10.1186/gb-2012-13-1-415 PMID: 22293439
17. Shiloh MU, Ruan J, Nathan C (1997) Evaluation of bacterial survival and phagocyte function with a fluorescence-based microplate assay. Infect Immun 65: 3193–3198. PMID: 9234774
18. Mariscal A, Lopez-Gigosos RM, Carnero-Varo M, Fernandez-Crehuet J (2009) Fluorescent assay based on resazurin for detection of activity of disinfectants against bacterial biofilm. Appl Microbiol Biotechnol 82: 773–783. doi: 10.1007/s00253-009-1879-x PMID: 19198831
19. Mak PA, Santos GF, Masterman K-A, Janes J, Wacknov B, et al. (2011) Development of an automated, high-throughput bactericidal assay that measures cellular respiration as a survival readout for Neisseria meningitidis. Clin Vaccine Immunol 18: 1252–1260. doi: 10.1128/CVI.05028-11 PMID: 21715580
20. Chen X, Niyonsaba F, Ushio H, Okuda D, Nagaoka I, et al. (2005) Synergistic effect of antibacterial agents human beta-defensins, cathelicidin LL-37 and lysozyme against Staphylococcus aureus and Escherichia coli. J Dermatol Sci 40: 123–132. doi: 10.1016/j.jdermsci.2005.03.014 PMID: 15963694

21. Nagaoka I, Kuwahara-Arai K, Tamura H, Hiramatsu K, Hirata M (2005) Augmentation of the bactericidal activities of human cathelicidin CAP18/LL-37-derived antimicrobial peptides by amino acid substitutions. Inflamm Res 54: 66–73. doi: 10.1007/s00011-004-1323-8 PMID: 15750713

22. Peñuelas-Urquides K, Villarreal-Treviño L, Silva-Ramírez B, Rivadeneyra-Espinoza L, Said-Fernández S, et al. (2013) Measuring of Mycobacterium tuberculosis growth. A correlation of the optical measurements with colony forming units. Braz J Microbiol 44: 287–289. Available: http://www.scielo.br/scielo.php?script = sci_arttext&pid=S1517-83822013000100042&lng = en&nrm = iso&tlng = en.

23. Okuda D, Yomogida S, Tamura H, Nagaoka I (2006) Determination of the antibacterial and lipopolysaccharide-neutralizing regions of guinea pig neutrophil cathelicidin peptide CAP11. Antimicrob Agents Chemother 50: 2602–2607. doi: 10.1128/AAC.00331-06 PMID: 16870748

24. Nakajima Y, Alvarez-Bravo J, Cho J, Homma K, Kanegasaki S, et al. (1997) Chemotherapeutic activity of synthetic antimicrobial peptides: correlation between chemotherapeutic activity and neutrophil-activating activity. FEBS Lett 415: 64–66. Available: http://linkinghub.elsevier.com/retrieve/pii/S0014579397011010. Accessed 2014 Aug 20. doi: 10.1016/S0014-5793(97)01101-0 PMID: 9326370

25. Tratnyek PG, Reilkoff TE, Lemon AW, Scherer MM, Balko BA, et al. (2001) Visualizing Redox Chemistry: Probing Environmental Oxidation?Reduction Reactions with Indicator Dyes. Chem Educ 6: 172–179. doi: 10.1007/s00897010471a

26. Olsson B, Gigante B, Mehlig K, Bergsten A, Leander K, et al. (2010) Apolipoprotein C-I genotype and serum levels of triglycerides, C-reactive protein and coronary heart disease. Metabolism 59: 1736–1741. Available: http://www.ncbi.nlm.nih.gov/pubmed/20580041. Accessed 2014 Aug 20. doi: 10.1016/j.metabol.2010.04.017 PMID: 20580041

27. Churg A, Dai J, Zay K, Karsan A, Hendricks R, et al. (2001) Alpha-1-antitrypsin and a broad spectrum metalloprotease inhibitor, RS113456, have similar acute anti-inflammatory effects. Lab Invest 81: 1119–1131. doi: 10.1038/labinvest.3780324 PMID: 11502863

28. Påhlman LI, Mörgelin M, Kasetty G, Olin a I, Schmidtchen a, et al. (2013) Antimicrobial activity of fibrinogen and fibrinogen-derived peptides—a novel link between coagulation and innate immunity. Thromb Haemost 109: 930–939. Available: http://www.ncbi.nlm.nih.gov/pubmed/23467586. Accessed 2014 Jun 6. doi: 10.1160/TH12-10-0739 PMID: 23467586

29. Rose G, Geselowitz A, Lesser G (1985) Hydrophobicity of amino acid residues in globular proteins. Science (80-) 229: 834–838. Available: http://www.sciencemag.org/content/229/4716/834.short. Accessed 2014 Jun 25. doi: 10.1126/science.4023714

30. Dean SN, Bishop BM, Van Hoek ML (2011) Natural and synthetic cathelicidin peptides with anti-microbial and anti-biofilm activity against Staphylococcus aureus. BMC Microbiol 11: 114. Available: http://www.ncbi.nlm.nih.gov/pubmed/21605457. doi: 10.1186/1471-2180-11-114 PMID: 21605457

31. Dean SN, Bishop BM, van Hoek ML (2011) Susceptibility of Pseudomonas aeruginosa Biofilm to Alpha-Helical Peptides: D-enantiomer of LL-37. Front Microbiol 2: 128. Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3131519&tool = pmcentrez&rendertype = abstract. Accessed 2014 Sep 26. doi: 10.3389/fmicb.2011.00128 PMID: 21772832