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Additivity and synergy between an antimicrobial peptide and inhibitory ions ☆

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ABSTRACT

Recently we described the pH dependence of activity for a family of cationic antimicrobial peptides (CAMPs) selected from a combinatorial library. In the current work we report on the effects of toxic ions (Cu^{2+}, Zn^{2+}, and F^-) and the chelator EDTA on the activity profiles of one member of this family, the 12-residue cationic antimicrobial peptide *ARVA, against a panel of microorganisms. All four ions exhibited either synergy or additivity with *ARVA for all organisms tested with the exception of *ARVA combined with NaF against *Candida albicans* which exhibited indifference. $CuCl_2$ and $ZnCl_2$ exhibited synergy with *ARVA against both the Gram negative *Pseudomonas aeruginosa* and the Gram positive *Staphylococcus aureus* as well as strong additivity against *Escherichia coli* at submillimolar concentrations. The chelator EDTA was synergistic with *ARVA against the two Gram negative organisms but showed only simple additivity with *S. aureus* and *C. albicans* despite their much lower MICs with EDTA. This effect may be related to the known differences in the divalent ion binding properties of the Gram negative LPS layer as compared to the peptidoglycan layer of the Gram positive organism. Unlike the other ions, NaF showed only additivity or indifference when combined with *ARVA and required much higher concentrations for activity. The yeast *C. albicans* did not show synergy or strong additivity with any of the inhibitory compounds tested. The effects of toxic ions and chelators observed here have important implications for applications using CAMPs and for the design of novel formulations involving CAMPs. This article is part of a Special Issue entitled: Interfacially Active Peptides and Proteins. Guest Editors: William C. Wimley and Kalina Hristova.

1. Introduction

Antibiotic resistance continues to be a significant problem with an increasing number of hospitalizations and deaths attributed to resistant organisms each year [1,2]. Developing new antibiotics and new formulations is essential to combating this threat. Cationic antimicrobial peptides (CAMPs) are potential drug candidates, having broad spectrum activity, high potency, and showing little tendency for microbial resistance [3–5].

CAMPs are a central component of the innate immune system in organisms ranging from invertebrates to plants to humans [6,7]. Most CAMPs share common characteristics such as low molecular weight and an amphipathic sequence containing both hydrophobic and cationic amino acids [3]. Most CAMPs or synthetic mimics appear to work by increasing the permeability of the cytoplasmic membranes of targeted organisms, although they may have additional intracellular targets [5,8–10]. The rarity of resistance to CAMPs is often ascribed to the lack of a distinct macromolecular target and to the large energetic cost in reorganizing the membrane surface of an organism [4,11].

The CAMP peptide family that contains *ARVA, designed to have β-sheet secondary structure in membranes, was selected from a combinatorial library [12] using lysis of a simple model membrane as a screen. Surprisingly, the peptides were found to have broad spectrum antimicrobial activity [13] despite the general nature of the screen. The primary structure of a promising member of this family, the peptide *ARVA, is described below. The peptides are named using the nomenclature adopted previously [13,14] to describe members of this family which contain a common 9-residue core motif with or without the presence of cationic terminal tripeptide cassettes. The asterisks designate the presence of a RRG- or -GRR terminal cassette and the one letter codes represent the amino acids found at each of the four varied positions (O) within the 9-residue core sequence, WOLOLOLOY. The peptides synthesized varied between 9 and 15 amino acids in length. The peptide used in this work, designated *ARVA, is a 12 amino acid peptide (RRGWALRLVLAY-NH_2) containing an N-terminal Arg–Arg–Gly cassette and containing A, R, V, and A respectively at the four varied (O) positions. The peptide is amidated at the C-terminus.

The initial step for CAMPs involves a binding event in which the positively charged peptides interact with the anionic plasma membranes of microorganisms [4,5]. In our previous study [14], we found

Abbreviations: MSCs, peptide minimum sterilizing concentrations; MICs, peptide minimum inhibiting concentrations; CAMPs, cationic antimicrobial peptides

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that pH dependent interactions with other charged molecules of the microbial cell wall or lipopolysaccharide layers [15–17] were also critical. Subsequent to binding of the plasma membrane, the amphipathic CAMPs interact with the nonpolar constituents of membrane lipids [7] and cause cell death due to membrane disruption via interfacial activity or a related mechanism rather than by pore formation although other mechanisms have also been proposed [11].

Due to the common difficulties associated with peptide drugs (e.g. solubility, cost, degradation), the most likely applications for CAMPs will be in topical or surface sterilization applications or to limit hospital-acquired infections [3,18]. Diabetic ulcers and other skin infections such as thrush, as well as infections related to cystic fibrosis have been the subject of several clinical trials [19] and new candidates have recently appeared [20].

Since it seems likely that CAMPs will often be used in situations where the environment can be varied such as in ointments, mouthwashes, and lavages, it is important to thoroughly understand the effects of environmental factors on the efficiency of CAMPs in directly killing bacteria [18,20]. A number of studies have looked at the effects of pH or salt concentration on the ability of CAMPs to kill microorganisms [21,22]. For example, it is well known that higher salt concentrations often interfere with CAMP activity and may be clinically relevant [23–25]. In our previous work [14] we investigated the effect of a wide range of pH and ionic strength values on CAMP activity.

Metals and toxic ions have been used in medicine and agriculture since antiquity [26–28] and new applications such as in combating biofilms and improved wound care continue to be explored [29–31]. Heavy metals in particular have a long history of use in medicine and have recently garnered renewed interest in the form of nanoparticle and modified surface technologies [32,33]. Zinc and copper are essential micronutrients for most cells but are toxic to prokaryotic cells at submillimolar concentrations [34–36] and to eukaryotic cells at higher concentrations or in ion transport diseases [37,38]. More than 30 copper proteins have been identified in higher organisms, but many fewer are found in bacteria and some bacteria appear to have no copper proteins. As a consequence, bacteria often do not contain ion transporters capable of importing copper and instead rely on pumps to export excess copper and, in some cases, binding proteins to maintain copper homeostasis [34,36]. Traditionally the toxic effects of copper have been attributed to Fenton type reactions creating reactive oxygen species such as H_2O_2 in the cytoplasm (oxidative stress) or to depletion of cellular sulfhydryl species [36] but more recent evidence points toward disruption of iron–sulfur proteins by displacement of iron by copper [34,36].

Zinc, on the other hand, is found in a greater number of proteins in both prokaryotes and eukaryotes and most bacteria do have transporters that import zinc as needed. The free concentrations for both metals, however, are maintained at extremely low concentrations in bacterial cells [34,36,38]. Zinc is often used in oral care products [39,40] and has been found to have a number of bacteriostatic effects at submillimolar concentrations against a variety of aerobic and anaerobic organisms. Zinc enhances the membrane permeability of protons and thus interferes with F-ATPase activity and in addition is an inhibitor of glycolytic enzymes [39,41]. Disruption of the proton gradient and glycolysis results in a reduction of the acid secretion often implicated in cariogenesis. Zinc toxicity for bacteria such as *S. pneumoniae*, on the other hand, appears to be related to competition with import of other essential cation species such as Mn(II) [35]. While some AMPs, such as the anionic dermcidin derived peptides found in human sweat [42], or the histidine containing demegen from saliva [43] require zinc for activity, this is not the case for the cationic *ARVA which contains no histidines.

Toxic or bacteriostatic anions such as EDTA and NaF are commonly used in the food industry [44], in oral health applications [28,39,45,46], and in the medical technology industry [31,47,48], often in prophylactic formulations. In many organisms, fluoride is toxic only at relatively high concentrations. One of the earliest reports (in 1903), for example, found that *Saccharomyces cerevisiae* was killed by 250 mM NaF (~4500 ppm) [28]. By comparison, drinking water is often fluoridated to a level of 1 ppm while most commercial toothpastes contain ~1000 ppm fluoride. Fluoride acts through a variety of effects including enzyme toxicity, mimicry of phosphate by metal–fluoride complexes, and strong inhibition of F-ATPases via enhanced proton permeability of membranes in the form of HF [28,39,49]. The effects on some oral bacterial species can occur in the micromolar to low millimolar (~1–200 ppm) concentration range, depending on pH, although anticariogenic effects are also related strongly to effects on the remineralization of tooth enamel [46,50].

Development of resistance to single drug treatments by microorganisms and cancerous cells is commonly encountered. To counter this, the use of multiple drug treatments has been adopted to improve the clinical outcomes for a variety of infective organisms as well as in chemotherapy [51–54]. Here we extend our work on environmental effects on antimicrobial peptide activity by testing for synergy between toxic ions and the synthetic CAMP *ARVA. We examined the effects of two toxic cations (Cu^{2+}, Zn^{2+}) and two toxic anions (EDTA, F^-) when used in combination with *ARVA.

2. Materials and methods

2.1. Bacterial cultures

Strains of *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853) and *Candida albicans* (ATCC 90028) were obtained from the American Type Culture Collection (Rockville, MD). The bacterial and yeast cultures were maintained in Trypticase soy broth (TSB) and YPD broth (Difco Laboratories, Detroit, Mich.) respectively. Overnight cultures of bacteria were grown in 50 mL tubes shaken at 220 rpm at 37 °C. Overnight cultures of yeast were grown at 30 °C.

2.2. Antimicrobial activity assays

Most buffer solutions and microbial suspensions were prepared in a liquid test medium (LTM) background containing 1% growth broth in 0.1 M MOPS at pH 7. $CuCl_2$, $ZnCl_2$, and *ARVA solutions were prepared in 0.025% acetic acid while NaF used LTM made with PBS. MOPS was used rather than phosphate in these studies as divalent ions will precipitate in the presence of phosphate. *E. coli*, *P. aeruginosa*, *S. aureus* and *C. albicans* were grown to mid-logarithmic phase and diluted to 10^4 colony forming units (CFU)/mL with minimal liquid test medium (LTM). Exposure time in minimal media was limited to avoid depleting nutrients prior to incubation. EDTA:*ARVA combinations in particular could give very low MSC values when cells were left for more than an hour in minimal media. We have shown that increasing the experimental cell counts up to 10^5/mL uniformly increases MSC values by several-fold, but does not affect the functional dependence of MSC measurements [13]. Assays were performed in sterile Costar 96-well flat bottom plates (Corning, Lowell, MA).

Preliminary MSC assays had a final assay volume of 200 μL containing 100 μL of cell suspension (2×10^4 CFU/mL in minimal LTM) added to 100 μL of 0.025% acetic acid containing various concentrations of toxic or inhibitory ions ($CuCl_2$, $ZnCl_2$, EDTA, NaF) or the peptide *ARVA that had been serially diluted (2-fold) across each row. Initial concentrations appropriate for each microbe being tested were added to the first well of each row before dilution. Typical starting concentrations were 10 μM for *ARVA, 500 mM for NaF, and 10–50 mM for $CuCl_2$, $ZnCl_2$, and EDTA. Rows were set up in pairs, with and without treatment, to control for bacterial growth in LTM. The well plates were then incubated at 37 °C for 30 min to allow killing to occur. Variation of the initial incubation time between 15 min and 1 h had no significant effect. Following this initial incubation step, 100 μL of 3× concentrated growth

medium at neutral pH was added to the cell suspension and cells were allowed to recover by overnight incubation at 37 °C.

Synergy assays (J. He and W. Wimley, personal communication, manuscript describing new synergy assay in preparation) were prepared in a similar fashion except that the reagents were added as "triplets" to sets of three columns and 2-fold serial dilutions were made down the column (Fig. 1). Synergy assays had a final assay volume of 200 μL containing 100 μL of cell suspension (2×10^4 cells/mL in minimal LTM) and 100 μL of containing various concentrations of toxic or inhibitory ions ($CuCl_2$, $ZnCl_2$, EDTA, NaF) and/or *ARVA in 0.025% acetic acid. Plates were prepared in the following manner. First, 100 μL of 0.025% acetic acid was added to all wells. The peptides and ions to be tested were then added to the top well in each column such that the total volume was 200 μL with additional 0.025% acetic acid added as necessary to reach the final volume. In each set of three columns, reagent A was added by itself at some multiple of MSC to the first column, reagent B was added similarly to the second column, and in the third column, both reagents A and B were added but at half the respective concentrations used in the first two columns. Typically, four replicates of such triplets were prepared in a single 96-well plate. Dilutions were then made down the columns by serially transferring 100 μL resulting in a final volume of 100 μL. A volume of 100 μL of LTM (0.1 M MOPS) containing bacterial suspensions (10^4 CFU/mL) was then added to each well and the plates incubated at 37 °C for 30 min followed by addition of 100 μL of 3× TSB growth media and overnight incubation at 37 °C.

Cell survival was evaluated by visual inspection as well as measuring optical density at 600 nm. Wells were either opaque (OD > 0.5) indicating stationary phase growth or they were transparent (OD < 0.02) indicating no growth (see Fig. 1). Very few wells had intermediate growth, but those that did were assigned a value of 0.5 columns killed. Aliquots from wells with no apparent growth were spread on nutrient agar plates to verify sterility and count colonies if present. In most cases there were few if any CFU in wells containing *ARVA compared to 10^8 CFU/μL in the opaque wells. EDTA was found to be mostly inhibitory (bacteriostatic) and wells plated for EDTA in the absence of *ARVA typically contained several thousand colonies, consistent with the number in the initial inoculum. $CuCl_2$ and $ZnCl_2$ sterilized at higher concentrations but often contained hundreds of colonies at lower concentrations in the absence of *ARVA.

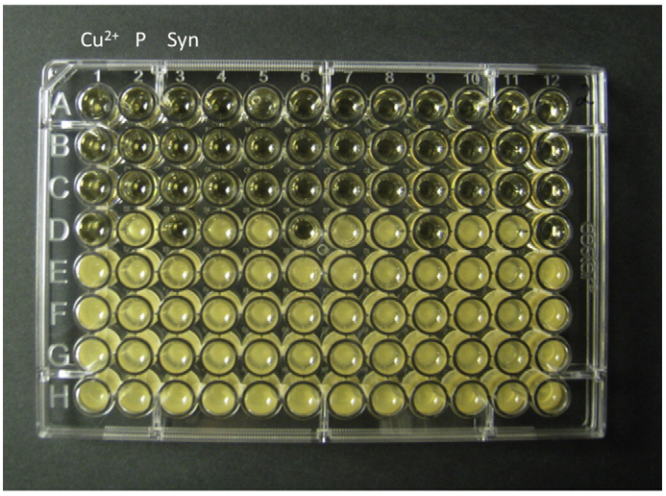

Fig. 1. Typical 96-well data acquired in these experiments. Data shown are for *ARVA in combination with $CuCl_2$ against *E. coli*. The 12 columns are prepared in sets of 3 columns. Columns 1, 4, 7, and 10 contain only $CuCl_2$ (Cu^{2+} in figure) at 4× MSC, columns 2, 5, 8, and 11 contain only the peptide *ARVA (P in figure) at 4× MSC and columns 3, 6, 9, 12 are the synergy columns (Syn in figure) containing both compounds at 2× MSC. The plates are sequentially diluted (2-fold) down each of the 8 rows of the plate. Opaque wells are overgrown with bacteria while clear wells indicate sterilization at that concentration.

2.3. Data analysis

For most experiments there were a number of sterile wells starting from the highest concentration of peptide. The lowest concentration of peptide that prevented cell growth is the minimum sterilizing concentration (MSC). For EDTA, the value should be considered a minimal inhibitory concentration (MIC). The MSC values were calculated by converting number of wells killed into a concentration value using the relation $MSC = C * D^{(n-1)}$ where C is the initial peptide concentration, D is the dilution factor for that experiment, and n is the number of wells killed. Reported MSC values are the average of 12–21 data points. Unless indicated otherwise, all error bars are ±1 standard error.

Synergy data were analyzed using the following [55,56] definitions:

$$FIC_A = MSC_{Ca} / MSC_A \text{ and } FIC_B = MSC_{Cb} / MSC_B$$

where FIC_A is the fractional inhibitory concentration for compound A defined as the ratio of the MSC for compound A when combined with compound B (MSC_{Ca}) divided by the MSC of compound A alone (MSC_A) and similarly for FIC_B. The MSC_C values were determined from the third (combined) column in each triplet as the average of 12–21 separate determinations as described above while the values for MSC_A and MSC_B were similarly determined from columns one and two of each triplet. The average MSC values were then used to calculate the values of FIC_A and FIC_B for each set of experiments. FIC_A and FIC_B are not constants as they can vary as the ratios of compounds A and B are varied. The value of the overall FIC value used to determine synergy or additivity is a constant designated FIC_C and is calculated simply as $FIC_C = FIC_A + FIC_B$. The following values of FIC_C are used to assign interactions: a value <0.5 indicates synergy, a value between 0.5 and 2.0 indicates additivity, a value between 2.0 and 4.0 indifference, and a value >4.0 antagonism [55,56]. Standard errors for FIC values were estimated from the family of FICs calculated individually using MSC data grouped in the same 96-well plates.

3. Results

3.1. Synergy assay

Synergy data were collected for the CAMP *ARVA against a panel of microorganisms as described in the Materials and methods section. In Fig. 1, the results for a typical synergy assay (in this case for *ARVA/$CuCl_2$ against *E. coli*) are displayed. The 12 columns are arranged in sets of three (triplets) in the following manner. In row A, columns 1, 4, 7, and 10 contain $CuCl_2$ at 4× MSC, columns 2, 5, 8, and 11 contain the peptide *ARVA at 4× MSC, and columns 3, 6, 9, and 12 contain both compounds at 2× MSC. Each row is diluted serially by 2-fold dilutions such that the concentrations in row B are 1/2 of row A and so on.

Following the addition of bacteria, the plates are incubated for 30 min, growth media added and the plates allowed to recover overnight at 37 °C (see the Materials and methods section). Each column contains clear wells indicating complete sterilization during the incubation period with no subsequent growth overnight. The columns containing $CuCl_2$ alone were completely sterilized down to a MSC value of ~500 μM (3–4 wells killed) while those containing *ARVA had an MSC of ~2.2 μM (3 wells killed). When combined at 2× MSC (column 3), killing was observed for 4 wells which corresponds to ~250 μM for $CuCl_2$ and 0.25 μM for *ARVA (see Table 1).

3.2. Effects of $CuCl_2$ and $ZnCl_2$

The results of synergy assays performed with all four microorganisms using *ARVA paired with $CuCl_2$ (Table 1) and $ZnCl_2$ (Supplementary Table S2) are given below. In each table, the number of separate determinations (n) is given along with the MSC concentrations and FIC values for each compound and combination. The tables are arranged

Table 1
Synergy data for *ARVA with $CuCl_2$.

Organism	MSC ± standard error (μM)								
	n	$[*ARVA]_C$	$[*ARVA]_A$	FIC_A	$[CuCl_2]_C$	$[CuCl_2]_B$	FIC_B	FIC_C	Interpretation
Gram negative									
P. aeruginosa	20	0.11 ± 0.02	2.9 ± 0.2	0.04 ± 0.02	136 ± 24	442 ± 120	0.31 ± 0.15	0.35 ± 0.15	Synergistic
E. coli	17	0.25 ± 0.07	2.2 ± 0.2	0.11 ± 0.04	246 ± 72	506 ± 110	0.49 ± 0.14	0.60 ± 0.16	Additive
Gram positive									
S. aureus	13	0.19 ± 0.07	3.9 ± 0.3	0.05 ± 0.02	930 ± 333	4500 ± 490	0.21 ± 0.08	0.26 ± 0.09	Synergistic
Yeast									
C. albicans	12	0.67 ± 0.07	1.8 ± 0.06	0.37 ± 0.04	3400 ± 340	4200 ± 350	0.81 ± 0.13	1.18 ± 0.17	Additive

such that the combined MSC value (subscript C) for each compound is listed first followed by the MSC value for that compound alone (subscript A for *ARVA and subscript B for the toxic ion) and by the individual FIC values for each compound. At the far right of each table, the combined FIC value (FIC_C) and interpretation are given.

The FIC_C values (±1 standard error) for each toxic ion paired with *ARVA against the panel of microorganisms are plotted in Fig. 2 while the individual FIC_A and FIC_B values for each combination are plotted in Fig. 3. Two sets of synergy assays conducted with varied starting ratios of *ARVA:$CuCl_2$ for *E. coli* are given in Supplementary Table S3 and plotted in Fig. 3 (open circles).

When *ARVA and $CuCl_2$ are used in combination, there are synergistic or strongly additive effects for all three bacteria tested. The concentration of *ARVA needed for complete sterilization in the presence of submillimolar concentrations of $CuCl_2$ (100–900 μM depending on microbe) is decreased by a factor of 9–25 fold down to submicromolar concentrations of *ARVA in the 100–250 nM range (Table 1). The resulting FIC_A values thus fall in the range from 0.04 to 0.11. The effect on the MSC of $CuCl_2$ is less pronounced. The two Gram negative species are already fairly sensitive to $CuCl_2$ with MSCs in the 400–500 μM range but in the presence of *ARVA they decrease roughly 2–3 fold. *S. aureus*, on the other hand, requires 4.5 mM $CuCl_2$ for sterilization, but in the presence of *ARVA this is reduced about 5-fold to less than 1 mM.

For *C. albicans*, the effect is additive but the resulting FIC_A value is about 0.37 while the FIC_B value is 0.81. The individual FIC values are plotted in Fig. 3 (filled circles) as FIC_B versus FIC_A to highlight the relative effects on the MSC of each compound when used in combination. Adding the two values (x and y coordinates) results in the FIC_C values which are given at the far right of Table 1 and also plotted in Fig. 2 (panels A through D). The FIC_C values for *P. aeruginosa* (Fig. 2B) and

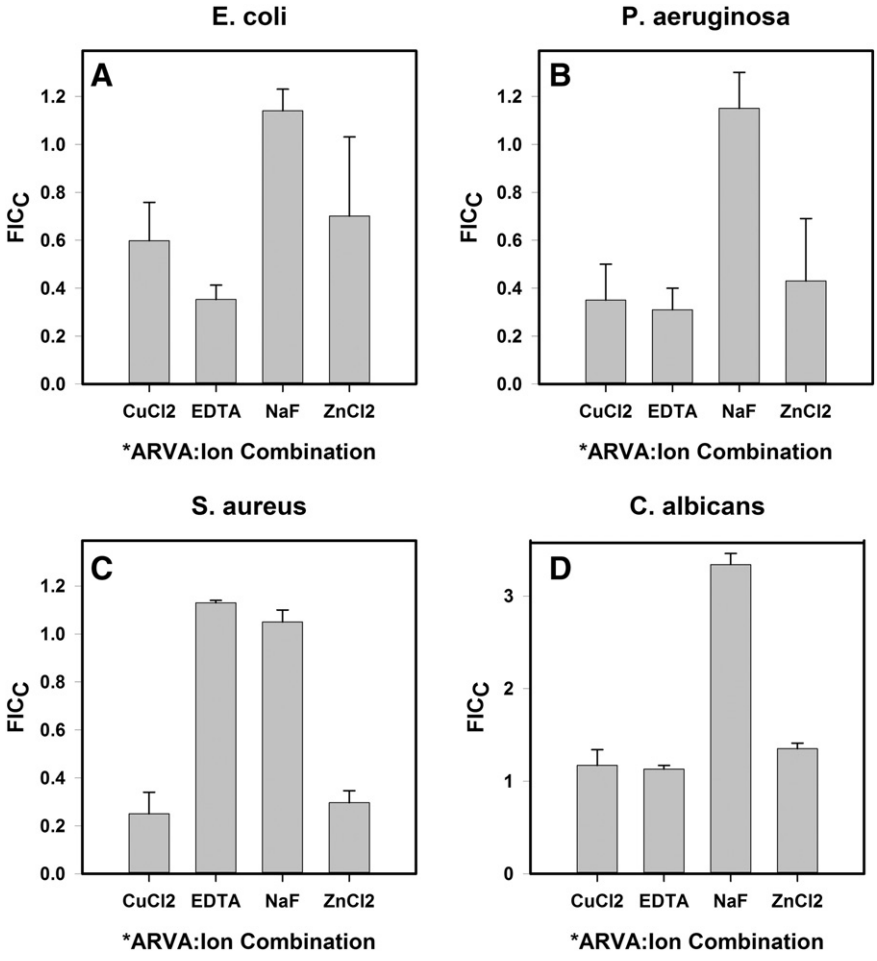

Fig. 2. The FIC_C values for *ARVA in combination with each ion are plotted. Panels A–D show data against *E. coli* (A), *P. aeruginosa* (B), *S. aureus* (C), and *C. albicans* (D). The FIC_C values are plotted a scale from 0 to 1.2 except for *C. albicans* which is plotted from 0 to 3.5.

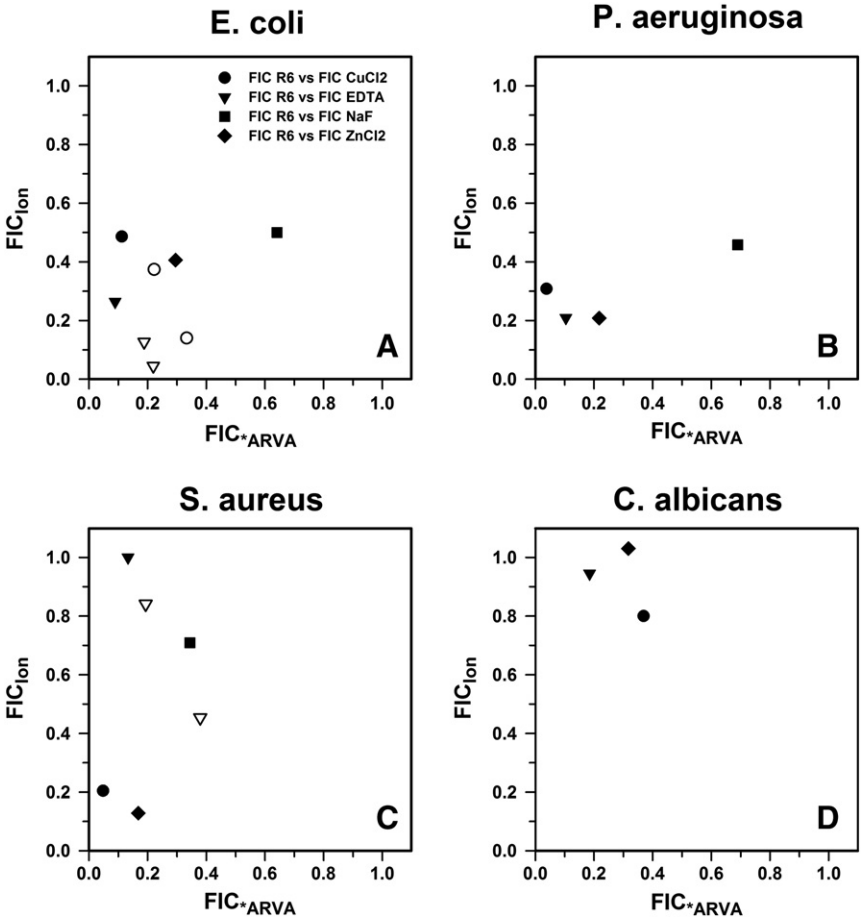

Fig. 3. Values for FIC_{Ion} are plotted versus FIC_{*ARVA} for each of the four toxic ions. $CuCl_2$ data are plotted as circles, EDTA as triangles, NaF as squares, and $ZnCl_2$ as diamonds. Data with filled symbols are from one set of peptide:ion ratios (Tables 1, 2, 4, and 5) while open symbols are data using altered ratios of peptide compared to the toxic ion (Table S3). Panels A–D show data collected against *E. coli* (A), *P. aeruginosa* (B), *S. aureus* (C), and *C. albicans* (D).

S. aureus (Fig. 2C) are well below 0.5 and thus indicate synergy between *ARVA and $CuCl_2$ while that for *E. coli* (Fig. 2A) is 0.6 which indicates strong additivity — in contrast to the *C. albicans* (Fig. 2D) value of 1.18 suggesting simple additivity. Given additivity or synergy, however, the values of FIC_A and FIC_B are a function of the concentration ratios chosen for the two species. To test this idea, we ran two additional sets of assays using *E. coli* with the *ARVA:$CuCl_2$ ratios varied (top of Table S3) and plotted these as open circles in Fig. 3A. By increasing the concentration of *ARVA while decreasing the concentration of $CuCl_2$ in 2-fold steps, we were able to decrease the FIC_B from about 0.5 down to 0.14 (for $CuCl_2$) while increasing the FIC_A from 0.11 to 0.33 for *ARVA. The FIC_C however changes much less than either of the individual FIC values, verifying the additive effect of the two compounds. Two of the values of FIC_C for *ARVA with $CuCl_2$ were nearly identical (~0.6) indicating strong additivity, but the third value of FIC_C of 0.47 suggests synergy, although all three values are consistent within standard error.

The data for synergy assays using *ARVA in combination with $ZnCl_2$ are given in Table S2 and FIC values plotted as before in Figs. 2 and 3 (diamond symbols). The data show a very similar pattern to that for $CuCl_2$ with all three bacteria showing a strong effect for $ZnCl_2$ while *C. albicans* exhibits simple additivity. Again *E. coli* (Fig. 2A) exhibits strong additivity while the FIC_C values for *P. aeruginosa* (Fig. 2B) and *S. aureus* (Fig. 2C) indicate synergy. The decrease in MSC for *ARVA is very similar for all four microbes in the presence of $ZnCl_2$ with all FIC_A values falling between 0.17 and 0.31 indicating a 3–6 fold decrease in the concentration of *ARVA needed for killing. In addition, all four MSC values for *ARVA were similar, falling in the 400–800 nM range. The concentrations of $ZnCl_2$ required were 2–5 fold lower than for $CuCl_2$ for both $ZnCl_2$ alone (200–1400 μM) and in the presence of *ARVA (50–170 μM) for the three bacteria but were more similar for *C. albicans*. The same pattern, with *S. aureus* less sensitive to the metal chloride in the absence of *ARVA but showing a much smaller FIC_B than the Gram negative organisms, was observed with $ZnCl_2$.

3.3. Effects of EDTA

The synergy assay data for EDTA in combination with ARVA is given in Table S4 and plotted in Figs. 2 and 3 (closed triangles). In addition, additional synergy data using varied ratios of *ARVA:EDTA were collected for both *E. coli* and *S. aureus* and are summarized in Table S3 and plotted in Fig. 3 as open triangles.

FIC_C values for both Gram negative organisms show synergy while that for *S. aureus* and *C. albicans* show simple additivity. However, the measured FIC_A values for all microorganisms tested are similar, exhibiting 5–10 fold decreases of MSC down to 250–500 nM *ARVA. The Gram negative organisms show a large effect on FIC_B as well with the MIC for EDTA decreasing 5-fold down to ~1 mM. At the original *ARVA:EDTA ratio (Table S4) the MSC for *ARVA decreased about 10-fold ($FIC_A = 0.09$) against *E. coli*, but at two different *ARVA:EDTA ratios (Table S3), the measured FIC_A value (*ARVA) increased 2-fold to ~0.20 (MSC ~ 500 nM) while the measured FIC_B value (EDTA) fell to as low as 0.05 indicating that the MIC can decrease by a factor of 20 down to ~200 μM EDTA in the presence of increased [*ARVA].

Interestingly, the MICs for EDTA for both *S. aureus* and *C. albicans* (300–600 μM) are already much lower than for the Gram negative organisms (4–5 mM) and decrease very little in the presence of *ARVA.

In Table S3, with varied ratios of *ARVA:EDTA for *S. aureus*, however, the FIC_B value for EDTA falls as low as 0.45, indicating that the [EDTA] necessary for killing in the presence of *ARVA can decrease from ~700 μM down to ~300 μM. The FIC_C decreases as well to 0.83 but still falls in the additive range.

3.4. Effects of NaF

Synergy data for NaF combined with *ARVA are given in Table S5 and plotted in Figs. 2 and 3 (square symbols). Unlike the other ions tested, no combination of NaF with *ARVA resulted in synergy or strong additivity for any of the organisms targeted. All three bacterial species did exhibit simple additivity however, resulting in FIC_A and FIC_B values in the range from 0.35 to 0.71 and FIC_C values near 1.0. As a consequence, the presence of ~50 mM NaF (~1000 ppm) reduced the MSC for *ARVA to near 1 μM. The effect was most substantial for *S. aureus* where the MSC decreased nearly 3-fold to ~1.4 μM *ARVA in the presence of ~70 mM NaF. The MSC values for NaF with the two Gram negative organisms decreased the most (~2-fold; FIC_B ~ 0.5) down to ~50 mM NaF.

The results for *ARVA:NaF against *C. albicans* were not promising with the MSC for NaF only decreasing by 35% in the presence of *ARVA, and the presence of ~200 mM NaF actually *increasing* the concentration of *ARVA required by nearly 3-fold. The resulting FIC_C value for *ARVA:NaF is 3.35 indicating that at these concentrations, the two compounds are acting independently (borderline antagonism) instead of additively due mainly to the strong effects on the MSC of *ARVA and thus FIC_A. Note that the scale of the y-axis in Fig. 2D is expanded to allow this value to be plotted and that the values for NaF with *ARVA are not plotted in Fig. 3D (see Table S5 instead).

4. Discussion

The search for new compounds and improved formulations to combat microbial infections is necessarily a continuous process in order to stay one step ahead of the acquisition of resistance by pathogenic microorganisms [18]. Cationic AMPs (CAMPs) have been studied extensively due to advantages such as broad specificity and lack of resistance, although these advantages are tempered by disadvantages such as cost, susceptibility to degradation, and possible toxicity common to peptide drugs [3,4]. Many anticipated uses of CAMPs are in areas such as topical or surface applications [3,6]. In fact, most clinical trials to date involve the treatment of skin infection or the use of CAMPs in preventing surface colonization and biofilms [18,20]. Such applications allow a wider variety of environmental conditions than is possible with systemic administration. The use of toxic metals and ions to combat microbial infections has received renewed interest recently in the areas of surface modification, nanoparticle applications, and combating biofilms, sometimes in combination with CAMPs [29,30,32,33,39,57]. A fuller understanding of the effects and interactions of environmental factors such as pH, ionic strength, specific ions, and surface properties on CAMP activity will facilitate the development of new applications of CAMPs and new formulations as potential drug therapies [14,18,20].

Combination therapies are a well established therapy method which often improve clinical outcomes and may lower the chances of developing resistance although the acquisition of multidrug resistance is an increasing problem [51,53,58–62]. Traditional target based drug discovery has had limited success in the development of new targets [63]. The microbial membrane represents a more diffuse target which does not involve the specific molecular recognition found with most other drug–target interactions that actually facilitate the development of resistance [4]. The membrane thus represents an underexploited target [64] and CAMPs or their mimics [8,9] are ideally suited to this purpose. Combination therapies can work well as long as there are no unfavorable or antagonistic interactions between the drugs that limit the effectiveness of one or both in the presence of the other [51,54]. If the two drugs show additivity or synergy, additional advantages accrue apart from those related to preventing development of resistance [59]. For example, if additivity or synergy allows lower doses of both drugs to be used, there are cost savings as well as the possibility of fewer side effects. In addition, if one compound is much cheaper than the other, as with *ARVA (or any peptide drug), then the relative ratios of the two compounds can be adjusted accordingly (see Table S3) to achieve the maximum therapeutic effect at the lowest cost. Measuring and characterizing additivity and synergy have a long and intricate history [51–54,58,65–67]. Here we have employed modified Loewe definitions of synergy, additivity, indifference, and antagonism [55,56] and have used a simple and direct assay to measure the efficiencies of microbial killing by individual and combined treatments involving toxic ions and the CAMP *ARVA. The results obtained here monitored killing following a brief incubation in minimal media, such as might occur immediately after topical application using a lavage solution. In future work, we plan to extend these studies to rich media and using a physiological solution such as saliva or blood plasma.

An advantage of the assay employed in these studies is that it is straight forward to obtain a large number of repetitions, and that alternate concentration ratios can be readily compared. In this study, we chose to analyze the FIC_A and FIC_B values separately (Fig. 3, Tables 1, S2–S5) and showed that by varying the *ARVA:ion concentration ratios (Table S3) we could tailor individual improvements in MSC values and thus the FIC_A and FIC_B values for each species.

A significant result of this work is that all three bacterial species, both Gram positive and Gram negative, showed either synergistic or strongly additive effects with the divalent ions Zn^{2+} or Cu^{2+} used in combination with *ARVA. The results for $CuCl_2$ (Table 1) indicated that the three bacterial species exhibited 9 to 25 fold improvements in the [*ARVA] required for sterilization (~100–250 nM). Although *E. coli* did not exhibit synergy at the original *ARVA:$CuCl_2$ concentration ratios (Table 1), at some ratios (Table S3), synergy was observed. This highlights the importance of varying the concentration ratios in such studies and in our view suggests that the advantages of additivity may sometimes be overlooked. In this case, though *E. coli* did not show synergy in most cases, sterilization was still achieved at 250 nM *ARVA in the presence of 250 μM $CuCl_2$.

The Gram positive *S. aureus* exhibited a stronger effect on both FIC_A for Cu^{2+} (filled circles, Fig. 3) and Zn^{2+} (diamonds, Fig. 3) and FIC_C (Fig. 2), particularly when compared to *E. coli* ($FIC_C = 0.26$ vs. 0.60 for $CuCl_2$, see also Tables 1 and S2). These differences could reflect the different types of metal binding sites found on the outer PG layer of *S. aureus* compared to the outer LPS layer of the Gram negative organisms [68–71] or alternately could simply reflect species or strain specific differences in metal homeostasis factors such as ion pumps and metal binding proteins (chaperonins) between the organisms [37,72]. Most eukaryotic organisms are less sensitive to the effects of toxic ions and can tolerate higher concentrations of such ions [28,37]. Since most microbes can readily develop resistance to heavy metals [72] the use of combination therapy can act to limit such resistance. At the same time, additivity or synergy can allow the use of lower concentrations of toxic ions, thus limiting potential side effects. As described above, *E. coli* showed complete sterilization in the presence of very low concentrations of both $CuCl_2$ and *ARVA (Table 1). With alternate ratios of *ARVA:$CuCl_2$, however, we observed sterilization at a [$CuCl_2$] as low as 60 μM in the presence of 940 nM *ARVA (Table S3). Thus one could choose to optimize the [*ARVA] to control costs, or alternately, could choose to lower the [$CuCl_2$] to limit ion toxicity. The results for $ZnCl_2$ (Table S2) were also encouraging in that all four organisms were killed by *ARVA in the 500 nM range with the three bacterial species only requiring ~50–150 μM $ZnCl_2$ to achieve this result. The yeast *C. albicans*, by contrast, has an MSC for $ZnCl_2$ as well as for $CuCl_2$ in the 3–4 mM range (see Tables 1 and S2).

In addition to synergy with divalent ions, we observed that both Gram negative species show strong synergy with EDTA paired with

*ARVA while the Gram positive *S. aureus* shows only simple additivity (Fig. 2, Table S4). Relying uncritically on definitions such as synergy and additivity, however, can result in missing potentially significant improvements in activity. For example, we observed that while the effect for *S. aureus* was only additive, the [EDTA] needed was ~600 μM compared to about twice that for the two Gram negative organisms and that the improvement to the MSC of *ARVA (FIC_A) was nearly identical for all three bacteria. In addition by varying the *ARVA:EDTA ratio (see Table S3), we could observe effects as low as 200–300 μM EDTA for all three organisms with FIC_A values between 0.2 and 0.4, thus still allowing substantially less of the comparatively expensive *ARVA to be used.

Gram negative organisms contain both an outer lipopolysaccharide (LPS) layer and a thin proteoglycan (PG) layer external to the plasma membrane while Gram positive organisms have only a very thick outer PG layer [73]. Both cell wall outer layers contain charged molecules that bind metal ions and strongly influence CAMP binding [14]. The thick PG layers of most Gram positive bacteria give them a large capacity to bind metal ions [71]. They can do so using both the anionic carboxyl groups of their PG layer as well as the anionic phosphoryl groups of their many wall and membrane bound teichoic acids [74]. The binding strengths, however, are often somewhat lower for Gram positive organisms due to the involvement of carboxyl ligands [68,71,75] compared to the stronger metal binding by Gram negative organisms using mostly phosphate groups of their LPS layer as ligands [69,75]. Such differences in binding capacity and binding affinity may be related to the different concentration regimes we observed in these studies (Tables S3 and S4) for EDTA toxicity between the Gram positive *S. aureus* and the Gram negative *E. coli* and *P. aeruginosa*.

The mechanism for toxicity for EDTA and other similar molecules is most likely related to their primary activity which is metal chelation. By starving growing bacteria of divalent metals such as Ca^{2+} and Mg^{2+}, and micronutrients such as Zn^{2+}, Cu^{2+}, and Mn^{2+}, EDTA can act in a bacteriostatic fashion to prevent bacterial growth. In addition, EDTA can also remove Ca^{2+}, and Mg^{2+} from their binding sites in bacterial cell walls, which function to both store metals for future use and to crosslink and strengthen bacterial cell walls [73,76,77]. Indeed, EDTA in combination with amine buffers has long been known to have toxic effects on bacteria [78]. More recently, EDTA has been employed to limit biofilms [30], to prevent infections and sterilize catheters [47,48], and in combinations with CAMPs or other antimicrobials [23,79,80].

In general, for compounds exhibiting simple additivity, no unique mechanism of toxicity need be invoked. For compounds exhibiting strong additivity or synergy, however, it is reasonable to infer that an additional interaction between the two compounds or an additional mechanism for toxicity must exist. The reason for synergy between the toxic metals (Zn^{2+}, Cu^{2+}) and *ARVA is unknown but may be related to the ability of the antimicrobial peptide to increase the permeability of the membranes to the small metal ions. An additional mechanism which can explain enhanced activity and synergy between EDTA and cationic compounds such as *ARVA is that upon removal of divalent metal ions from the bacterial cell walls, the cell walls become much more anionic and thus attract the cationic molecules much more strongly. We recently highlighted the importance of charges on bacterial cell walls for CAMP action including the ability of high pH to increase negative charge on *S. aureus* cell walls, and the potential role of modifications that neutralize charge on cell walls in infectivity by pathogenic organisms [14].

In these studies, NaF did not show synergy or strong additivity with *ARVA (Table S5) but did show simple additivity for the three bacterial species. The Gram negative species showed only a small decrease in the [*ARVA] required but were still killed at close to 1 μM *ARVA in the presence of ~60 mM NaF. *S. aureus* exhibited a larger decrease (~3-fold) in [*ARVA] needed down to 1.4 μM in the presence of ~70 mM NaF. Given that many eukaryotic cells can tolerate much higher concentrations of NaF [28], even the above additive improvements could be beneficial. Mai et al. [80], however, have observed synergy between 10 ppm NaF and a CAMP against the oral bacteria *Streptococcus mutans*. The results for *Candida* are discussed below.

Results obtained for *C. albicans* were generally not as promising as with the three bacterial species, but this is probably explained by the better tolerances to metals and other toxic compounds exhibited generally by eukaryotes. In particular, eukaryotic organisms have a greater variety of ion pumps, chaperonins, and other metal binding proteins such as the metallothioneins [28,37]. As observed with results for the bacterial species, however, simply relying on FIC_C alone and the accompanying interpretation may mask important information. With both $CuCl_2$ and $ZnCl_2$ (Tables 1 and S2), for example, we observed a 3-fold improvement (FIC_A ~ 0.33) in the [*ARVA] required for sterilization of *C. albicans*. This resulted in MSC values in the 600–700 nM range for *ARVA in the presence of 3–4 mM metal ions.

Surprisingly, the results obtained with *ARVA:EDTA for *C. albicans* (Table S4) while characterized as simple additivity were actually as good or better than the synergism observed for the two Gram negative species. All three were able to sterilize at concentrations of *ARVA of ~250 nM but 1 mM EDTA was required for this effect for the two Gram negative organisms while with *Candida* this result required only 274 μM EDTA. In addition, Wei et al. [23] have observed synergy between MUC7, derived from saliva, and EDTA against a pathogenic strain of *C. albicans*. The only treatment that did not result in synergy or additivity was the combination of *ARVA:NaF against *Candida*. In fact, we observed indifference or borderline antagonism between these two reagents. This effect is most likely due to ionic strength as the greater tolerance for NaF exhibited by *C. albicans* results in >200 mM NaF being present in the assay. High concentrations of salts are well known to impair function of CAMPs by screening the cationic peptides from the anionic components of the target molecules on the microbial surface.

In this study, we analyzed, in detail, the fractional inhibitory concentrations of both partners in the synergy assays. By doing so, we hoped to highlight the potential cost savings that can result for *ARVA or other peptide drugs even in cases of simple additivity. The use of low concentrations of relatively inexpensive sources of toxic ions such as simple salts like $ZnCl_2$ or $CuCl_2$, NaF, or Na_2EDTA could help to lower the costs for CAMP drugs. An additional advantage is that these ions are already approved and used in many other applications in food, agriculture, and medicine. Our results on the additive and synergistic effects of toxic ions when used in combination with the CAMP *ARVA may have important clinical implications for the development of future treatments and formulations involving antimicrobial peptides.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2014.05.005.

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