Adv Exp Med Biol - Advances in Microbiology, Infectious Diseases and Public Health (2019) 13: 43–59
https://doi.org/10.1007/5584_2018_276

Published online: 30 September 2018

Biological Profiling of Coleoptericins and Coleoptericin-Like Antimicrobial Peptides from the Invasive Harlequin Ladybird *Harmonia axyridis*

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Abstract

The spread of antibiotic-resistant human pathogens and the declining number of novel antibiotics in the development pipeline is a global challenge that has fueled the demand for alternative options. The search for novel drug candidates has expanded to include not only antibiotics but also adjuvants capable of restoring antibiotic susceptibility in multidrug-resistant (MDR) pathogens. Insect-derived antimicrobial peptides (AMPs) can potentially fulfil both of these functions. We tested two coleoptericins and one coleoptericin-like peptides from the invasive harlequin ladybird *Harmonia axyridis* against a panel of human pathogens. The AMPs displayed little or no activity when tested alone but were active even against clinical MDR isolates of the Gram-negative ESKAPE strains when tested in combination with polymyxin derivatives, such as the reserve antibiotic colistin, at levels below the minimal inhibitory concentration. Assuming intracellular targets of the AMPs, our data indicate that colistin potentiates the activity of the AMPs. All three AMPs achieved good in vitro therapeutic indices and high intrahepatic stability but low plasma stability, suggesting they could be developed as adjuvants for topical delivery or administration by inhalation for anti-infective therapy to reduce the necessary dose of colistin (and thus its side effects) or to prevent development of colistin resistance in MDR pathogens.

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Keywords
Anti-infectives · Antimicrobial peptides · Biological profiling · Coleoptericin · *Harmonia axyridis*

1 Introduction

The increasing prevalence of multidrug-resistant (MDR) bacteria and the lack of novel antibiotics in the development pipeline are a challenge to healthcare systems worldwide and have prompted the search for new antibiotic candidates, especially those active against Gram-negative bacteria (access to Medicine Foundation 2018; Delaney and Butter 2018; O'Neill 2016; Stern et al. 2017; WHO 2017). One promising class of candidates are the antimicrobial peptides (AMPs), which are produced by most if not all eukaryotic organisms but are particularly diverse and extensive among insects (Tonk and Vilcinskas 2017). Many families of insect AMPs demonstrate promising activity against human pathogens, e.g., certain insect cecropins (cationic, α-helical linear peptides) show potent in vivo activity against MDR *Acinetobacter baumannii* (Jayamani et al. 2015) and certain insect defensins (globular peptides with β-sheets stabilized by intramolecular disulfide bridges) are active against MDR *Staphylococcus aureus* (Li et al. 2017).

In insects with the most extensive AMP repertoires, there is evidence that multiple AMPs are co-expressed in response to infection and they interact to maximize their combined activity in vitro and in vivo (Pöppel et al. 2015). Beneficial combinatorial AMP interactions include potentiation (one AMP enabling or enhancing the activity of others) or synergy (the combined antimicrobial effects are greater than the sum of the individual activities). This enhances the efficacy of antimicrobial immune responses and reduces the resources reallocated to the innate immune system by increasing the antimicrobial activity of AMPs at lower concentrations (Rahnamaeian et al. 2016). The achievement of robust antimicrobial responses by the co-expression of AMPs with distinct modes of action explains why some insect-derived AMPs show little or no detectable antimicrobial activity when tested alone (Bolouri Moghaddam et al. 2016). This natural principle can be translated to medical applications, i.e., several insect-derived AMPs have been shown to interact synergistically with conventional antibiotics, suggesting they could be used to restore antibiotic sensitivity in MDR pathogens. For example, a cecropin produced by the mosquito *Aedes aegypti* was recently shown to act synergistically with tetracycline against *Pseudomonas aeruginosa*, which is responsible for most hospital-acquired diseases (Zheng et al. 2017). Similarly, a defensin from the beetle *Tribolium castaneum* was shown to act synergistically with telavancin and daptomycin against MDR *S. aureus* (Rajamuthiah et al. 2015).

Here we present the first biological profile of coleoptericins and coleoptericin-like peptides, which are specific for beetles (Coleoptera), from the harlequin ladybird *Harmonia axyridis* against a panel of human pathogens (Mylonakis et al. 2016). We selected several candidates from this species, which is native to Central and Eastern Asia but which has been introduced as a biological control agent in Northern America and Europe (Koch and Costamagna 2017; Roy et al. 2016). In the past two decades, it has become an invasive species that successfully outcompetes native ladybird species in the newly colonized areas (Roy et al. 2016), partly due to its superior immune system (Verheggen et al. 2017). *H. axyridis* constitutively produces an antibacterial and antiparasitic alkaloid called harmonine (Rohrich et al. 2012; Schmidtberg et al. 2013) but also carries inducible genes for up to 49 AMPs (Vilcinskas et al. 2013), which is much more extensive than the 15 genes found in the native seven-spotted ladybird *Coccinella septempunctata* and the 10 genes of the two-spotted ladybird *Adalia bipunctata* (Vogel et al. 2017). During the evolution of *H. axyridis*, the defensin and coleoptericin gene families have undergone unprecedented expansion, with 14 coleoptericins in *H. axyridis* but only 2 in *C. septempunctata* and 3 in *A. bipunctata* (Vogel et al. 2017). Another feature of the immune system which differs remarkably

among these three species is the maximum induction levels of some AMPs following a bacterial challenge, with the response in *H. axyridis* several orders of magnitude higher than in the two native ladybird species (Vogel et al. 2017). Remarkably, we discovered recently that coleoptericin1 (Col1) also shows population-specific expression patterns in *H. axyridis*, with invasive populations expressing higher maximum levels of Col1 than noninvasive populations. When the *col1* gene is silenced by RNA interference, *H. axyridis* becomes more susceptible to its natural pathogen *Pseudomonas entomophila*, but this susceptibility can be reversed by the injection of a synthetic Col1 peptide (Gegner et al. 2018). Taken together, these results inspired us to determine the activity of synthetic analogs of coleoptericins and coleoptericin-like peptides from *H. axyridis* against a panel of human pathogens. In addition, we investigated whether these beetle-derived AMPs displayed combinatorial activity with the peptide-based reserve antibiotic colistin, which was abandoned in the 1970s because of its severe side effects but is now being reintroduced due to the lack of alternative treatment options (Kelesidis and Falagas 2015; Tangden and Giske 2015).

2 Materials and Methods

2.1 Coleoptericins and Coleoptericin-Like Peptides

The amino acid sequences of the peptides Col1, Col6, and ColLC as well as their natural occurring derivatives Col4 (Col1 derivative), Col15 (Col6 derivative), and ColLA (ColLC derivative) are listed in Table 1. While Col1, Col4, Col6, and Col15 belong to the coleoptericin-type peptides, ColLA and ColLC are coleoptericin-like peptides. A sequence alignment, performed with the COBALT algorithm (Papadopoulos and Agarwala 2007), is depicted in Fig. S1. The peptides were produced by solid-phase synthesis on a polymeric carrier resin (GenScript, Piscataway, NJ, USA). They were analyzed by reversed-phase chromatography on a 4.6 × 250 mm Alltech Alltima C18 column (Thermo Fischer Scientific, Waltham, MI, USA) with an ascending acetonitrile gradient in water in the presence of a small amount of trifluoroacetic acid (0.05–0.065%). The peptides were detected by measuring the UV absorption at 220 nm as well as by electrospray ionization mass spectrometry (ESI-MS). The peptide purity was at least 90%.

2.2 Biological Isolates and Culture Conditions

The microbial isolates that were tested against the *H. axyridis* peptides are listed in Table S1. They were obtained from the American Type Culture Collection (Manassas, VA, USA) or the German Collection of Microorganisms and Cell Cultures GmbH (Braunschweig, Germany). Furthermore, meropenem-resistant and colistin-resistant clinical isolates derived from hospitalized patients in Germany were provided by Dr. Yvonne Pfeifer (Robert Koch Institute (RKI), Wernigerode, Germany). These isolates were identified by a RKI strain number. All isolates were cultivated in cation-adjusted Mueller-Hinton broth (CAMB) or (*Mycobacterium smegmatis* only) in brain heart infusion (BHI) medium supplemented with 1% Tween-80. All isolates were cultivated at 37 °C and 85% relative humidity, shaking at 180 rpm, and merely *Candida albicans* was cultivated at 28 °C. The meropenem-resistant and colistin-resistant isolates were maintained in the presence of the appropriate antibiotic at below the minimal inhibitory concentration (MIC).

2.3 Antibacterial Profiling

2.3.1 Inhibition of Bacterial Growth

MIC values were determined as previously described (Balouiri et al. 2016). Briefly, most of the bacterial test strains were grown for 18 h, whereas *M. smegmatis* and the yeast *C. albicans* were grown for 48 h. The cultures were subsequently diluted in CAMB medium to a final concentration of 5×10^5 cells/mL (most bacteria), 1×10^5 cells/mL (*M. smegmatis*), or 1×10^6

Table 1 Properties of the synthetic *H. axyridis* AMPs

AMP	Sequence	Size	MW (g/mol)	pI	Charge	G
Col1 BR022 Coleoptericin-type	SLQPGAPNFPIPGQEKQEGWKFDPSLTRGEDGNTLGSINIHHTGPNHEVGANWDKVIRGPGKAKPTYSIHGSWRW	75	8256.01	9.31	1.4	−0.985
Col4 BR023 Coleoptericin-type	SLQPGAPNFPMPGQGKQEGWKFDPSLTRGEDGNTLGSINIHHTGRNHEVGANWDKVIRGPGKAKPTYSIHGSWRW	75	8261.1	10.26	3.4	−1.017
Col6 BR021 Coleoptericin-type	SLQPGAPKLPYAWSRKQEGWKFDPSLTRGEDGNTLGSINIHHTGRNHEVGANWNKVIRGPGKAKPTYSIHGSWRW	75	8433.31	10.73	6.4	−1.032
Col15 Coleoptericin-type	SLQPGAPKLPYAWSGKQEGWKFDPSLTRGEDDNTLGSINIHHTGPNHEVGANWDKVIRGPGKAKPTYSIHGSWRW	75	8334.13	9.71	2.4	−0.98
ColLA	DTEGWKVQPNINRDQDGNTAGSVRVQKDFGNHEVHAGASKVFSGPNRGEPSYNVGATFNW	60	6544.91	6.08	−0.8	−1.037
ColLC	DSEGWKVQPNINRDQDGNTAGSVRVQKQLGNHEVHAGASRVFSGPNRGGPSYNVGATFNW	60	6465.86	9.69	1.2	−0.98

The AMP properties molecular weight (MW), isoelectric point (pI), and net charge at pH 7 (charge) were calculated using software provided at http://pepcalc.com/
G, GRAVY score, total hydropathy values of all the amino acids divided by the size (Kyte and Doolittle 1982)

cells/mL (*C. albicans*). Peptides and the control antibiotics tetracycline, gentamicin, meropenem, and colistin were dissolved in sterile water. The final test concentrations were 1,024–0.031 μg/mL for the peptides and 64–0.002 μg/mL for the control antibiotics. Testing was conducted in lidded 384-well plates in a test volume of 20 μL per well at 37 °C, 85% relative humidity, and 180 rpm. After incubation for 18 h, microbial growth was quantified by measuring the turbidity at 600 nm for most of the bacterial strains and *C. albicans*, and by luminometric ATP quantification using the BacTiter-Glo assay kit (Promega, Fitchburg, WI, USA) for *M. smegmatis*. Growth inhibition was calculated with respect to blank and growth control values, and the lowest AMP/antibiotic concentrations associated with no visible growth represented the MIC (the MIC of the control antibiotics was used to confirm the integrity of each assay). Experiments were performed as triplicates. To obtain preliminary results for AMP-colistin interaction studies, the MIC values of the AMPs were determined in the presence of 0.075 μg/mL colistin. To investigate effects of other polymyxin derivatives on the activity of the AMPs, the MICs of the AMPs were determined in the presence of sub-inhibitory concentrations (1/8 MIC) of each derivative.

2.3.2 Checkerboard Assay

The checkerboard dilution test of the AMPs with colistin was conducted in 96-well round-bottom microtiter plates in a final volume of 100 μL per well and a final bacterial density of 5×10^5 cells/mL. We set up a tenfold 1:2 serial dilution series of colistin in the range 320–0.31 μg/mL along each row from column 1–10 and a sixfold 1:2 serial dilution series of the AMP in the range 320–5 μg/mL down each column from row A to G on one assay master plate. Horizontal wells H1 to H11 were used for MIC testing of colistin and vertical wells A12 to G12 for MIC testing of the AMPs. We transferred 10 μL of each dilution from the assay master plate to an assay plate and added 90 μL of each bacterial suspension. Lidded plates were incubated for 18 h at 37 °C and 85% relative humidity shaking at 180 rpm and bacterial growth/growth inhibition was monitored visually. Experiments were performed in duplicates. The fractional inhibitory concentration (FIC) and the FIC_{index} of each AMP-colistin combination were calculated using the following formulae:

FIC for compound A= MIC of compound A in combination/MIC of compound A.

FIC for compound B = MIC of compound B in combination/MIC of compound B.

FIC_{index} = FIC A+ FIC B.

$FIC_{index} \leq 0.5$ indicate synergy. $FIC_{index} > 4$ indicates antagonism.

2.4 Toxicity Studies

2.4.1 Hemolysis of Human Erythrocytes

The hemolytic activity of the AMPs was tested in a 96-well round-bottom microtiter plate in a final volume of 100 μL. Erythrocytes were isolated from fresh citrate-stabilized blood from human donors by repeated centrifugation (5 min at 500x g) and washing with PBS. To obtain the final suspension, the isolated erythrocytes were diluted 1:50 in PBS. The peptides were dissolved in sterile water, and we prepared a threefold 1:2 dilution series in the concentration range 2048–256 μg/mL in a volume of 50 μL. We then added 50 μL of the erythrocyte suspension to each well, and the lidded test plates were incubated at 37 °C and 85% relative humidity for 5 h, shaking at 180 rpm. The erythrocytes were then pelleted and 80 μL of the supernatant was transferred to a new 96-well microtiter plate to quantify the released hemoglobin by turbidity measurement at 540 nm. The percentage hemolysis caused by the peptides was calculated relative to the values of the blank and positive control (Triton X-100).

2.4.2 Cytotoxicity Assay Based on ATP Quantification and the Uptake of Neutral Red

The toxicity of the AMPs toward the human hepatocellular carcinoma HepG2 HB-8065 (ATCC) cells was assessed by using the CellTiter-Glo ATP Monitoring Kit (Promega)

and by quantifying the ability to store the dye neutral red (NRU-solution, Sigma-Aldrich, St Louis, MI, USA). The assay was conducted in 96-well microtiter plates in a test volume of 200 µL. Peptides were tested in an eightfold 1:2 dilution series and a final concentration range of 400–1.56 µM. HepG2 cells were maintained in DMEM-F12 medium containing 1% nonessential amino acids, 1% sodium pyruvate, and 10% heat-inactivated fetal calf serum at 37 °C and 5% CO_2. Prior to each test, 100 µL of culture medium was added per well (each containing about 20,000 cells) and the plates were incubated for 16 h as above. The peptides were diluted in culture medium to obtain appropriate concentrations and were added to the wells as six replicates. Ketoconazole was used as a positive control for toxicity and PBS was used as the blank. After incubation for 24 h as above, cell viability was calculated either by cell lysis and subsequent luminometric quantification of the ATP concentration in each sample or by measuring the amount of neural red taken up by the cells. NRU uptake was measured at 540 nm (Tecan Genios Pro) after 3 h incubation with NRU solution and subsequent cell lysis. The stated no observed effect concentration (NOEC) values refer to the highest sample concentration with a cell viability >80%.

2.4.3 Inhibition of the Human Ether-a-go-go-Related Gene Potassium Channel

The effect of the coleoptericins and coleoptericin-like AMPs on the human ether-a-go-go-related gene (hERG) potassium channel was investigated using an automated patch-clamp method as described by (Houtmann et al. 2017). Peptides were diluted in a fivefold 1:3 dilution series at a final concentration range of 30–0.12 µM in extracellular medium (150 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose, 0.06% Pluronic F-68, 0.3% residual DMSO). The hERG channel was constitutively expressed in Chinese hamster ovary cells (CHO hERG Duo®, B'SYS GmbH, Witterswil, Switzerland). CHO cells were grown at a concentration of 8×10^6 CHO cells/mL in QPlates® (Sophion/Biolin Scientific, Ballerup, Denmark) in Ex-Cell® animal component-free CHO medium (Sigma-Aldrich) supplemented with 25 mM HEPES, 100 U/mL penicillin-streptomycin, and 0.004% soybean trypsin inhibitor. To each well, we added extracellular medium containing the desired concentration of AMPs. The peptide-hERG interaction was quantified by recording the tail current following repolarization of the hERG channels using a QPatch HTX station (Sophion/Biolin Scientific). The half-maximal inhibitory concentrations (IC_{50}) were determined using the values from three replicates of the AMP concentration series with respect to the terfenadine citrate positive control and extracellular medium (blank).

2.5 Stability Studies

2.5.1 Plasma Stability

Peptides were incubated at a final concentration of 5 µM in human, mouse, and rat plasma. After incubation at 37 °C for 0, 1, 4, and 24 h, 100 µL of the plasma samples were mixed with ethanol containing 0.5% (v/v) NH_3 to interrupt interactions between the AMPs and plasma proteins, and the latter were precipitated by centrifugation at 1735 x g for 20 min. Each 10 µL of the supernatant was analyzed in triplicates for the presence of Col6, Col1, or ColLC by LC-MS^2 (Q Exactive hybrid quadrupole-Orbitrap device, Thermo Fisher Scientific) using an AERIS Peptide 3.6 µm XB-C18 50 × 2.1 mm column (Phenomenex, Aschaffenburg, Germany). Acetonitrile and water solvents (supplemented with 0.1% formic acid) were used in an ascending acetonitrile gradient (flow rate = 500µL/min). The stability of each peptide was determined by comparing the peptide-specific ion peaks in the sample with the corresponding blank controls.

2.5.2 Metabolic Stability

The in vitro metabolic stability of the AMPs was determined using HMCS3S cryopreserved human hepatocytes (Thermo Fisher Scientific), which were stored in liquid nitrogen, thawed in cryopreserved hepatocytes recovery medium

(Thermo Fisher Scientific), and diluted to 5×10^5 cells/mL in William's E medium (Sigma-Aldrich) containing 0.001% dexamethasone and 4% cell maintenance supplement pack B (Thermo Fisher Scientific). Peptides were incubated in duplicates at 38 °C, 10% CO_2, and a final concentration of 1 μM for 0, 15, 30, 60, 90, and 120 min. After each time point, incubation was terminated by the addition of acetonitrile, the hepatocytes were removed by centrifugation, and the samples were analyzed by LC-MS^2 to detect the remaining peptides. The scaled predicted hepatic clearance for humans, as well as the extraction ratio, was calculated based on the peptide half-life assuming a liver weight of 25.71 g/kg body weight, hepatocellularity of 99×10^6 cells/g liver, and a hepatic blood flow of 1.24 L/h/kg (Poulin et al. 2012).

3 Results

3.1 Antimicrobial Activity Against Reference Strains

We investigated the potential antimicrobial activity of the coleoptericin and coleoptericin-like AMPs by testing Col1 and ColLC against selected Gram-positive bacteria (*S. aureus* ATCC 25923, *S. aureus* ATCC 33592, *Staphylococcus epidermidis* ATCC 35984, *Enterococcus faecium* DSM 17050, and *Listeria monocytogenes* DSM 20600) and Gram-negative bacteria (*E. coli* ATCC 25922, *Klebsiella pneumoniae* DSM 30104, *A. baumannii* ATCC 19606, *P. aeruginosa* ATCC 27853, and *Proteus mirabilis* DSM 4479), as well as *M. smegmatis* ATCC 607 and the yeast *C. albicans* FH2173 (Table S2). All peptides substantially lacked activity (MIC ≥1,024 μg/ml). In addition, Col6 was tested against *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *K. pneumoniae* DSM 30104, *A. baumannii* ATCC 19606, and *P. aeruginosa* ATCC 27853. Weak activity was observed against *E. coli, K. pneumoniae*, and *A. baumannii* (MIC = 32 μg/ml). There was no observed activity against *P. aeruginosa* (MIC = 256 μg/ml) or *S. aureus* (MIC >1,024 μg/ml). Three closely related natural derivatives of the aforementioned AMPs – namely, Col4 (Col1-derivative), Col15 (Col6 derivative), and ColLA (ColLC derivative) – were tested against *E. coli* ATCC 25922, and no antimicrobial activity was observed at concentrations up to 1,024 μg/mL (data not shown).

3.2 Interaction with Membrane-Disrupting Compounds

Given that the selected coleoptericin and coleoptericin-like AMPs play an important role in the *H. axyridis* immune system (Schmidtberg et al. 2013; Vilcinskas et al. 2013) but did not exhibit antimicrobial activity when tested alone, we hypothesized that they naturally act in combination with other insect-derived membrane-disrupting peptides. The peptide-based antibiotic colistin is known for its ability to disrupt bacterial membranes, so we tested Col1, Col6, and ColLC in combination with sub-MIC concentrations of colistin in order to explore this hypothesis. We therefore exposed selected Gram-positive bacteria (*S. aureus* ATCC 25923, *S. epidermidis* ATCC 35984, *E. faecium* DSM 17050, and *Listeria monocytogenes* DSM 20600) and Gram-negative bacteria (*E. coli* ATCC 25922, *E. coli* RKI 131/08, *E. coli* RKI 6A-6, *K. pneumoniae* DSM 30104, *K. pneumoniae* RKI 93/10, *K. pneumoniae* RKI 19/16, *A. baumannii* ATCC 19606, *A. baumannii* RKI 19/09, P. *aeruginosa* ATCC 27853, and *P. aeruginosa* RKI 93/12) to the AMP-colistin combination (Table 2). Against Gram-positive isolates, colistin-resistant isolates, *P. aeruginosa*, and one clinical *K. pneumoniae* isolate, the MIC of the AMPs were not affected. In contrast, the MIC of the weakly active Col6 decreased by 8–16-fold to 4 μg/mL for *E. coli* and *K. pneumoniae*, and by two–fourfold to 8 μg/mL for *A. baumannii*. The MIC of Col1 and ColLC decreased by at least 128-fold to 4–8 μg/mL for *E. coli, K. pneumoniae*, and a clinical isolate of *A. baumannii*. Against the wild-type *A. baumannii* strain, the MIC of Col1 was reduced by 16-fold to 32 μg/mL, whereas the MIC of ColLC was not affected. To investigate the interaction between colistin and the AMPs in

Table 2 Activity of the *H. axyridis* peptides in combination with colistin

	MIC (μg/ml)							
	CST	MEM	Col1		Col6		ColLC	
Strain	CAMB	CAMB	CAMB	+CST	CAMB	+CST	CAMB	+CST
E. coli ATCC 25922	0.5	0.13	>1024	4	64	4	>1024	4
E. coli RKI 131/08	0.5	>64	>1024	4	32	4	>1024	4
E. coli RKI 6A-6	8	0.13	>1024	>1024	128	8	>1024	>1024
K. pneumoniae DSM 30104	0.5	0.25	>1024	8	32	4	>1024	8
K. pneumoniae RKI 93/10	0.5	>64	>1024	>1024	64	nd	>1024	>1024
K. pneumoniae RKI 19/16	64	0.25	>1024	>1024	>1024	>1024	>1024	>1024
P. aeruginosa ATCC 27853	0.5	1	>1024	>1024	256	512	>1024	>1024
P. aeruginosa RKI 93/12	1	64	>1024	>1024	>1024	>1024	>1024	>1024
A. baumannii ATCC 19606	1	2	512	32	32	8	>1024	>1024
A. baumannii RKI 19/09	0.5	64	512	4	16	8	>1024	4

MIC values were determined in cation-adjusted Mueller-Hinton broth (*CAMB*) and in CAMB supplemented with 0.075 μg/ml colistin (+*CST*). MIC values of the control antibiotics colistin (*CST*) and meropenem (*MEM*) are listed
nd not determined

more detail, checkerboard assays against *E. coli*, *P. aeruginosa*, and *A. baumannii* were carried out using different dilutions of colistin paired with different dilutions of Col1 or ColLC. We observed AMP-colistin synergy for all combinations ($FIC_{index} \leq 0.5$), but the synergy was more pronounced for Col1 than ColLC (Fig. 1a, b). Further checkerboard assays were prepared with the addition of 150 mM NaCl or 1.25 mM $CaCl_2$. The presence of NaCl reduced the FIC_{index} by two–fourfold (Fig. 1c, d). The AMP-colistin interaction was non-synergistic in the presence of 1.25 mM $CaCl_2$ when tested against *P. aeruginosa*.

3.3 Interaction Between AMPs and Polymyxin Derivatives

We conducted a preliminary structure-activity relationship (SAR) study on the AMP-colistin interaction by testing Col1, Col6, ColLC, and the naturally occurring derivatives Col4 (derivative of Col1), Col15 (derivative of Col6), and ColLA (derivative of ColLC) combined with sub-MIC concentrations (1/8 MIC) of polymyxin derivatives against *E. coli* ATCC 25922. The natural derivatives Col4, Col15, and ColLA were inactive against *E. coli* ATCC 25922 (MIC >1,024 μg/mL). Eight different polymyxin B and seven different polymyxin E (colistin) derivatives (some unpublished) were tested. Although the activity of ColLA was not affected by the polymyxin derivatives, the activity of ColLC was reduced by at least 256-fold in the presence of colistin E2 (0.032 μg/mL) to 2 μg/mL and by at least 16-fold in the presence of polymyxin B (0.063 μg/mL) to 32 μg/mL (Table 3). In the presence of polymyxin B (0.063 μg/mL) or colistin E2 (0.032 μg/mL), the activity of the coleoptericins was enhanced (16-fold for Col6 and at least 128-fold for Col1, Col4, and Col15) to MIC values of 2–4 μg/mL. Furthermore, the activity of Col1, Col4, and Col15 was enhanced at least 4–16-fold by colistin E1 (0.063 μg/mL), the inactive polymyxin B decapeptide derivative A000160918 (32 μg/mL), and the colistin decapeptide analog A000500146A (0.125 μg/mL) to 32–128 μg/mL. In contrast, the activity of Col6 was only enhanced twofold by the inactive polymyxin B decapeptide derivative A000160918 (32 μg/mL) and both eightfold by colistin E1 (0.063 μg/mL) and the colistin decapeptide analog A000500146A (0.125 μg/mL), resulting in MIC values of 4 and 16 μg/mL. The other ten derivatives we tested did not affect the MICs of the AMPs (data not shown).

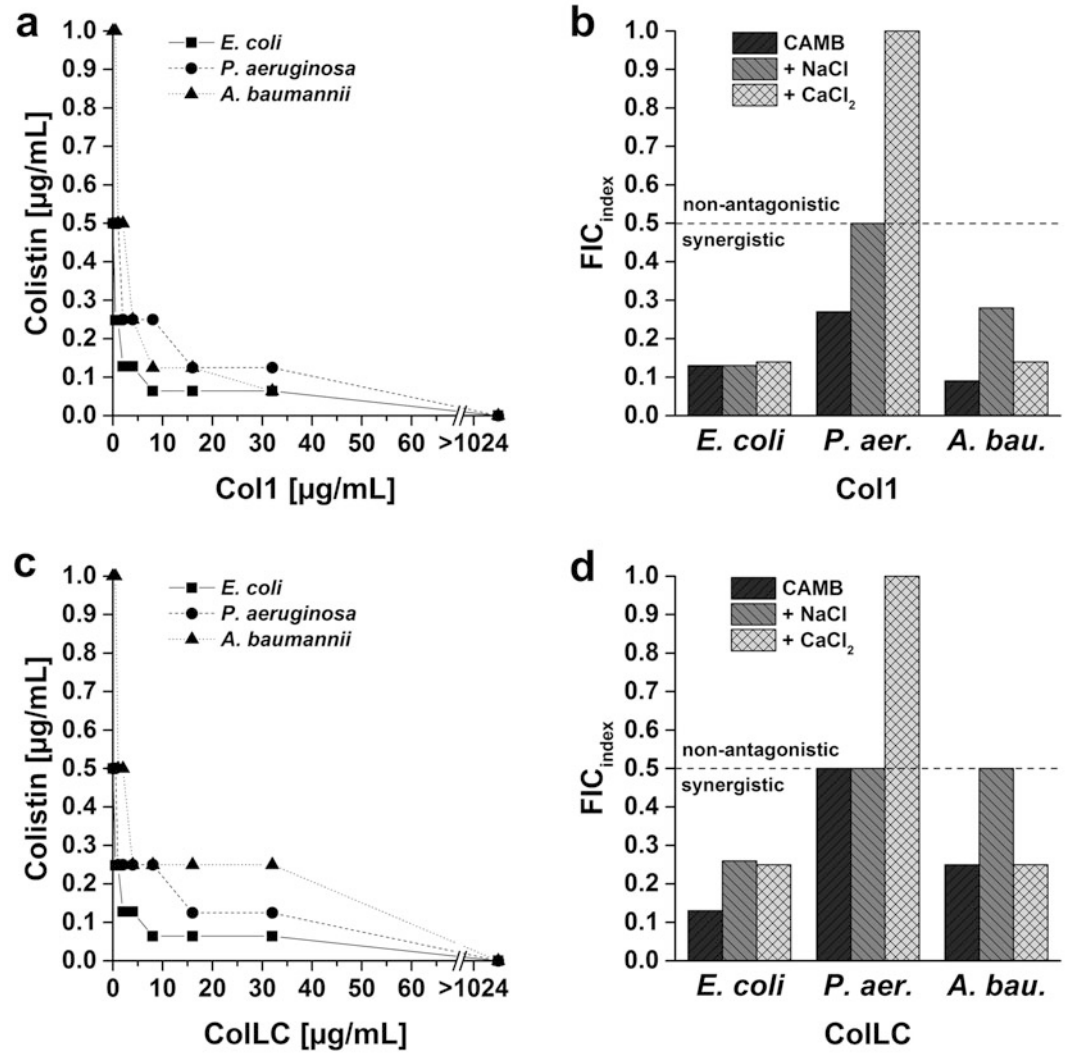

Fig. 1 Interaction of colistin with (**a**) Col1 and (**c**) ColLC in cation-adjusted Mueller-Hinton broth (*CAMB*) against *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *A. baumannii* ATCC 19606 depicted as isobolograms. Resulting FIC_{index} values were calculated for (**b**) Col1 and (**d**) ColLC in CAMB, in CAMB adjusted to 150 mM NaCl (*+NaCl*), and in CAMB adjusted to 1.25 mM $CaCl_2$ (*+$CaCl_2$*) for *E. coli*, *P. aeruginosa* (*P. aer.*), and *A. baumannii* (*A. bau.*). FIC_{index} values below 0.5 indicate synergy

Table 3 Activity of *H. axyridis* peptides in combination with polymyxin derivatives

Supplement		MIC [µg/mL]					
Cpd. Name	MIC/used conc. [µg/mL]	Col1	Col4	Col6	Col15	ColLA	ColLC
–		>256	>256	32	>256	>256	>256
Colistin E2	0.25/0.032	4	4	2	4	>256	2
Polymyxin B	0.5/0.063	4	2	2	4	>256	32
Colistin E1	0.5/0.063	64	32	4	64	>256	>256
A000500146A	1/0.125	64	64	4	64	>256	>256
A000160918	256/32	128	64	16	64	>256	>256

MIC values against *E. coli* ATCC 25922 were determined in cation-adjusted Mueller-Hinton broth (CAMB) and in CAMB supplemented with sub-MIC concentrations (1/8 MIC) of different polymyxin derivatives. MIC values of the supplemented polymyxin derivatives as well as the used sub-MIC concentrations are listed

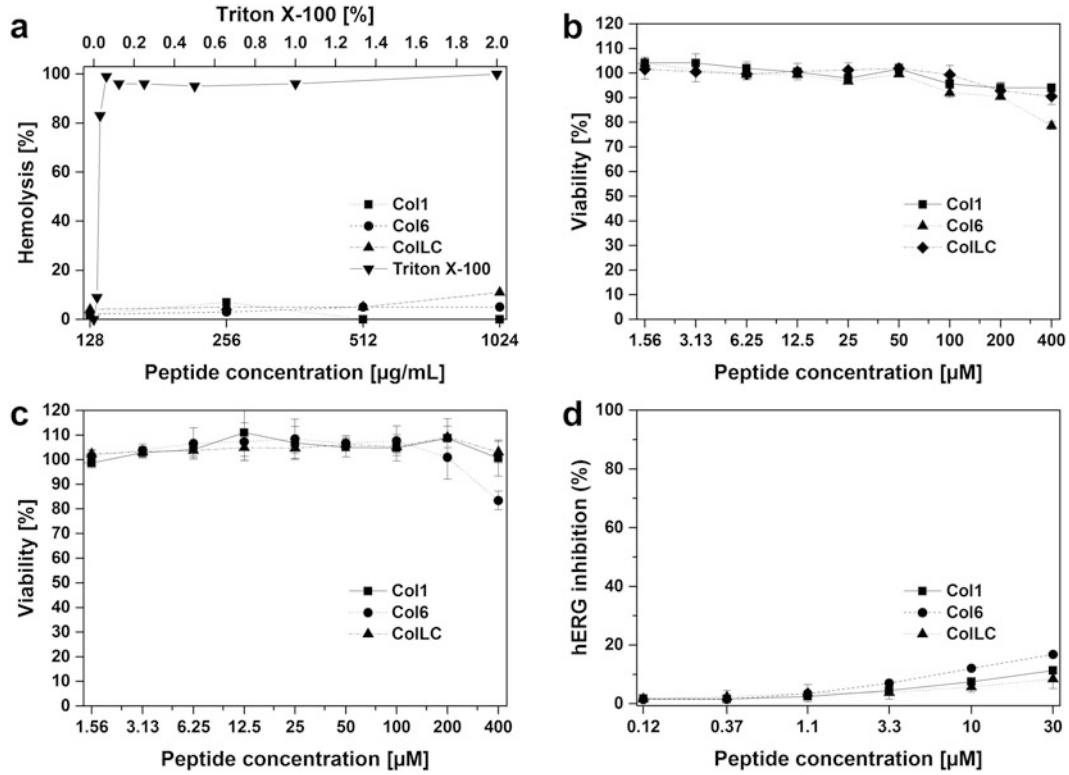

Fig. 2 Toxicity profiling of the *H. axyridis* coleoptericins Col1, Col6, and the coleoptericin-like AMP ColLC. (**a**) Hemolytic activity against human erythrocytes. Cytotoxic effects of on HepG2 cells were evaluated by measuring (**b**) neutral red uptake and (**c**) the concentration of ATP. (**d**) Inhibitory effects against the important off-target human ERG potassium channel

3.4 Toxicity Studies

The suitability of the coleoptericins and coleoptericin-like AMPs as adjuvants to minimize the dose of colistin for systemic administration in humans was investigated by toxicity assessment. First we tested the ability of Col1, Col6, and ColLC to disrupt the membrane of human erythrocytes (Fig. 2a). None of the peptides displayed hemolytic activity up to a concentration of 512 µg/mL. Next, we tested the toxicity of Col1, Col6, and ColLC toward HepG2 human hepatocellular carcinoma cells (Fig. 2b, c). The NOEC (cell viability >80%) was 100–400 µM (843–3,304 µg/mL), indicating that the peptides can be considered as nontoxic. To broaden the toxicity profile of the peptides, we used QPatch technology to test the antagonistic activity of Col1, Col6, and ColLC against the hERG potassium channel, an important off-target in the development of drugs for systemic administration in humans. No target-specific activity was observed, with IC_{50} values >30 µM (Fig. 2d).

3.5 Stability Studies

The metabolic stability of the coleoptericins and coleoptericin-like AMPs was tested in human hepatocytes. Col1, Col6, and ColLC were considered to be stable. The half-life of Col1 was 1,240 min in hepatocytes, resulting in a scaled human predicted hepatic clearance (hCL_{SP}) of 0.0672 L/h/kg and a human hepatic extraction ratio (Eh) of 12.1%. Col6 and ColLC showed no instability, preventing the calculation of hCL_{SP} and Eh values. The plasma stability of Col1,

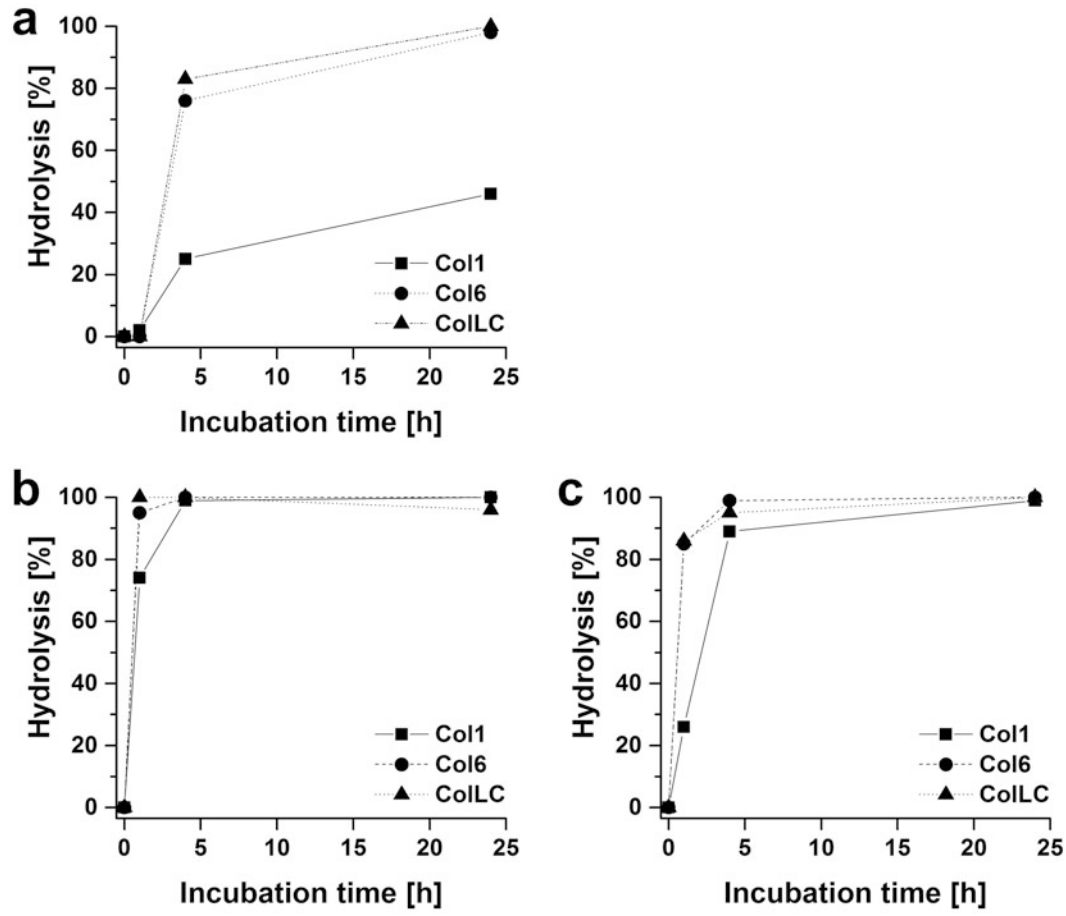

Fig. 3 Stability of the coleoptericins and coleoptericin-like AMPs Col1, Col6, and ColLC in plasma. Values indicate the percent hydrolysis of the *H. axyridis* peptides in (**a**) human, (**b**) mouse, and (**c**) rat plasma

Col6, and ColLC was tested with incubation periods of 1, 4, and 24 h (Fig. 3). All peptides were hydrolyzed after 4 h in all three plasma types (human, mouse, and rat). After 1 h, the peptides remained stable only in human plasma.

4 Discussion

Coleoptericins and coleoptericin-like peptides are glycine- and proline-rich AMPs that are structurally similar to the attacins but are found only in beetles (Mylonakis et al. 2016). They have been reported to operate in the control of endosymbionts rather than pathogen killing (Login et al. 2011; Masson et al. 2016). The deduced amino acid sequences of the *H. axyridis* coleoptericins include a signal peptide for extracellular localization, a furin cleavage site, and a mature peptide of ~75 amino acids (Vilcinskas et al. 2013). We selected two *H. axyridis* coleoptericins and one coleoptericin-like peptide for biological profiling against human pathogens based on several promising characteristics: (i) the number of genes encoding coleoptericins and coleoptericin-like peptides has expanded much more in *H. axyridis* than in native ladybirds, suggesting the peptides have undergone rapid functional diversification (Vilcinskas et al. 2013), (ii) Col1 is upregulated more than 10,000-fold in response to injected bacteria (Vilcinskas et al. 2013), (iii) Col1 is

expressed more strongly in invasive populations of *H. axyridis* than in noninvasive populations, and (iv) RNAi silencing of Col1 makes *H. axyridis* more susceptible to the entomopathogen *P. entomophila* but resistance can be restored by the injection of synthetic Col1 along with the bacteria (Gegner et al. 2018).

Surprisingly, the three AMPs showed little or no activity against human pathogens when tested alone. However, having previously shown that these coleoptericins potentiate the activity of *H. axyridis* c-type lysozymes against bacteria (Beckert et al. 2015), we postulated that their binding to intracellular targets in bacteria requires the simultaneous presence of membrane-disrupting compounds. The molecular mechanism underlying the potentiating functional interactions among insect-derived AMPs to increase their combined potency against Gram-negative bacteria was elucidated by combining abaecin and hymenoptaecin from the bumblebee *Bombus terrestris* (Rahnamaeian et al. 2015). The authors provided evidence that hymenoptaecin compromises the *E. coli* membrane in a manner that enables abaecin to enter the bacterial cell and interact with the bacterial chaperone DnaK, an evolutionarily conserved central organizer of the bacterial chaperone network.

To exploit the potentiating activity of coleoptericins for the development of new therapies, we tested coleoptericins and coleoptericin-like peptides combined with the peptide-based antibiotic colistin, which is used mostly as a reserve antibiotic due to its negative side effects (Falagas et al. 2005; Kelesidis and Falagas 2015). We confirmed that the coleoptericins and coleoptericin-like peptides were potentiated in the presence of colistin, increasing their activity against human pathogens, even including Gram-negative MDR clinical isolates. However, the potentiating effects with colistin were only observed against colistin-sensitive isolates. In preliminary experiments we also combined the peptides with the antibiotics meropenem, gentamicin, tobramycin, tigecycline, and rifampicin but did not observe effects on the resulting MIC values of the test bacterial strain. This supports the theory that colistin compromises the cell envelopes of Gram-negative bacteria and allows the coleoptericins to reach their intracellular targets. Similar effects were observed for hymenoptaecin from the bumblebee *Bombus terrestris*, which compromises the cell envelop of Gram-negative bacteria for abaecin (Rahnamaeian et al. 2015). Based on the experiments with colistin, we anticipated that mixtures of polymyxin B and the *H. axyridis* AMPs would also inhibit selected human pathogens. Notably, the polymyxin B nonapeptide is known to compromise the membranes of Gram-negative bacteria (Dixon and Chopra 1986; Vaara et al. 1984), but we found that it did not have any effect in combination with the AMPs, which is contrary to a pure membrane compromising role of the polymyxins in the polymyxin-AMPs interaction. To obtain preliminary SARs on the AMP-colistin interaction, we tested Col1, Col6, and ColLC as well as three derivatives of the coleoptericins and coleoptericin-like AMPs (Col4 (derivative of Col1), Col15 (derivative of Col6), and ColLA (derivative of ColLC)) combined with sub-MIC concentrations (1/8 MIC) of various polymyxin derivatives against *E. coli* ATCC 25922. While the activity of coleoptericins was similarly potentiated by colistin E2, colistin E1, polymyxin B, and two other polymyxin derivatives, the activity of ColLC was only potentiated by colistin E2 and polymyxin B. ColLA did not show activity in any tested combination. The coleoptericin-like AMPs clearly differ by length from the coleoptericins, and there are also significant charge differences (Fig. S1). Using the cobalt algorithm for alignment, it is noticeable that, on position 16, the coleoptericins are positively charged, whereas the coleoptericin-like AMPs are negatively charged. At positions 41, 58, 70, and 74, the coleoptericins are positively charged and the coleoptericin-like AMPs uncharged and at positions 39 and 50 it is vice versa. Furthermore, at positions 23 the coleoptericins are negatively charged and the coleoptericin-like AMPs are uncharged. Since bridging of the cell envelope is dependent on the charge of the compounds, this charge differences could explain the different

interaction patterns of the coleoptericins and the coleoptericin-like AMPs with the polymyxins. Charge may also explain why ColLA has not shown activity in any tested combination with the polymyxins. Unlike all other AMPs, ColLA has a negative charge at alignment position 43 while all others are uncharged at this position.

Because the antibacterial activity of various AMPs is known to be compromised by high concentrations of salt (Chu et al. 2013; Huang et al. 2011; Maisetta et al. 2008), we carried out checkerboard assays combining colistin and the *H. axyridis* AMPs under standard conditions in CAMB and in parallel in the same medium adjusted to 150 mM NaCl or 1.25 mM $CaCl_2$, approximately representing the salt concentration in human plasma (Li et al. 2016; Walser 1961). These assays revealed minimal salt sensitivity, so we proceeded to profile the AMPs under the rigorous standards of the pharmaceutical industry to assess whether these AMPs could be suitable as adjuvants in combination with colistin for systemic antibiotic therapy. One of the greatest barriers to the systemic use of AMPs is their potential toxicity to eukaryotic cells, particularly erythrocytes (Kang et al. 2014), which is associated with their high net charge and hydrophobicity (Laverty and Gilmore 2014; Teixeira et al. 2012). Col1, Col6, and ColLC showed neither hemolytic activity against erythrocytes nor toxicity toward HepG2 cells, which probably reflects the relatively low charge and hydrophobicity of these peptides (Table 1). Instability in body fluids is another vulnerability of AMPs for systemic administration (Chung et al. 2015; Diao and Meibohm 2013). We found that the *H. axyridis* AMPs were stable in human hepatocytes ($t_{1/2} > 1{,}200$ min) but unstable in human, mouse, and rat plasma. Overcoming the proteolytic degradation of AMPs or prolonging their half-life in serum is challenging because the activity of AMPs depends on their tertiary structure, and this limits the extent of chemical modifications to enhance stability (Rao et al. 2005). Indeed, strategies such as PEGylation, dendrimerization, pro-peptide administration, and cyclization can all extend the peptide half-life but must not inhibit the biological function (Brunetti et al. 2016; Knappe et al. 2010; Lam et al. 2016; Pini et al. 2005). The use of D-enantiomers can also extend the peptide half-life, but activity is lost (Casteels and Tempst 1994) reflecting the stereospecific nature of coleoptericin interactions with intracellular targets (Krizsan et al. 2015; Login et al. 2011). The low plasma stability of the natural peptides is incompatible with systemic in vivo delivery, but they could nevertheless serve as chemical scaffolds for the development of more stable analogs. In conclusion, due to their high in vitro therapeutic index and their potentiating activity with colistin against MDR Gram-negative bacteria, coleoptericins and coleoptericin-like peptides may be useful as leads for the development of adjuvants for topical delivery or administration by inhalation. Due to their multi-target activity in combination with polymyxins, and the resulting lower doses of polymyxins, coleoptericins and coleoptericin-like AMPs could prevent the emergence of pathogen strains that are resistant against polymyxin antibiotics.

5 Funding

This study was funded by the Hesse State Ministry of Higher Education, Research and the Arts (HMWK) via a generous grant for the LOEWE Center for Insect Biotechnology and Bioresources and by the Federal Ministry of Education and Research (BMBF) in Germany via the project "Triple-In."

Acknowledgments We thank Dr. Yvonne Pfeifer for providing the multidrug-resistant clinical isolates from the strain library of the Robert Koch Institute in Wernigerode. We thank Kirsten-Susann Bommersheim, Sibylle Müller-Bertling, and Kirstin Ganske for excellent technical assistance and Dr. Richard M. Twyman for professional editing of the manuscript.

Conflict of Interest The authors declare no conflict of interest.

Appendix

Table S1 Overview of the test strains and their culture conditions

Test strain	Properties	Culture medium	Temperature [°C]
Gram-negative bacteria			
E. coli ATCC 25922	Type strain	CAMB	37
E. coli RKI 131/08	Clinical isolate	CAMB	37
E. coli RKI 6A-6	Clinical isolate	CAMB	37
K. pneumoniae DSM 30104	Type strain	CAMB	37
K. pneumoniae RKI 93/10	Clinical isolate	CAMB	37
K. pneumoniae RKI 19/16	Clinical isolate	CAMB	37
P. aeruginosa ATCC 27853	Type strain	CAMB	37
P. aeruginosa RKI 93/12	Clinical isolate	CAMB	37
A. baumannii ATCC 19606	Quality control strain	CAMB	37
A. baumannii RKI 19/09	Clinical isolate	CAMB	37
P. mirabilis DSM 4479	Type strain	CAMB	37
Gram-positive bacteria			
S. aureus ATCC 25923	MSSA	CAMB	37
S. aureus ATCC 33592	MRSA	CAMB	37
S. epidermidis ATCC 35984	Clinical isolate	CAMB	37
E. faecium DSM 17050	VRE	CAMB	37
L. monocytogenes DSM 20600	Type strain	CAMB	37
M. smegmatis ATCC 607	Wild type	BHI + 1% Tween-80	37
C. albicans FH2173	Wild type	CAMB	28

CAMB cation-adjusted Mueller-Hinton broth, *BHI* brain heart infusion medium, *MSSA* methicillin-sensitive *S. aureus*, *MRSA* methicillin-resistant *S. aureus*, *VRE* vancomycin-resistant enterococci

Table S2 Activity of the *H. axyridis* AMPs against reference strains

	MIC (μg/ml)		
Strain	Col1	Col6	ColLC
S. aureus ATCC 25923	>1024	>1024	>1024
S. aureus ATCC 33592	>1024	nd	>1024
S. epidermidis ATCC 35984	>1024	nd	>1024
E. faecium DSM 17050	>1024	nd	>1024
L. monocytogenes DSM 20600	>1024	nd	>1024
E. coli ATCC 25922	>1024	32	>1024
K. pneumoniae DSM 30104	512	32	>1024
A. baumannii ATCC 19606	512	32	>1024
P. aeruginosa ATCC 27853	512	256	>1024
P. mirabilis DSM 4479	>1024	nd	>1024
M. smegmatis ATCC 607	>1024	nd	>1024
C. albicans FH2173	>1024	nd	>1024

MIC values were determined in cation-adjusted Mueller-Hinton broth (CAMB)
nd not determined

Fig. S1 Alignment of the coleoptericins and the coleoptericin-like AMPs. Amino acid differences that result in charge differences of coleoptericin-like to coleoptericins are marked in red font. Amino acid differences that affect charge differences between the coleoptericin-like AMPs ColLA and ColLC are marked in green font

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