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Profiling antimicrobial peptides from the medical maggot *Lucilia sericata* as potential antibiotics for MDR Gram-negative bacteria

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Background: The ability of MDR Gram-negative bacteria to evade even antibiotics of last resort is a severe global challenge. The development pipeline for conventional antibiotics cannot address this issue, but antimicrobial peptides (AMPs) offer an alternative solution.

Objectives: Two insect-derived AMPs (LS-sarcotoxin and LS-stomoxyn) were profiled to assess their suitability for systemic application in humans.

Methods: The peptides were tested against an extended panel of 114 clinical MDR Gram-negative bacterial isolates followed by time–kill analysis, interaction studies and assays to determine the likelihood of emerging resistance. In further *in vitro* studies we addressed cytotoxicity, cardiotoxicity and off-target interactions. In addition, an *in vivo* tolerability and pharmacokinetic study in mice was performed.

Results: LS-sarcotoxin and LS-stomoxyn showed potent and selective activity against Gram-negative bacteria and no cross-resistance with carbapenems, fluoroquinolones or aminoglycosides. Peptide concentrations of 4 or 8 mg/L inhibited 90% of the clinical MDR isolates of *Escherichia coli*, *Enterobacter cloacae*, *Acinetobacter baumannii* and *Salmonella enterica* isolates tested. The '*all*-D' homologues of the peptides displayed markedly reduced activity, indicating a chiral target. Pharmacological profiling revealed a good *in vitro* therapeutic index, no cytotoxicity or cardiotoxicity, an inconspicuous broad-panel off-target profile, and no acute toxicity in mice at 10 mg/kg. In mouse pharmacokinetic experiments LS-sarcotoxin and LS-stomoxyn plasma levels above the lower limit of quantification (1 and 0.25 mg/mL, respectively) were detected after 5 and 15 min, respectively.

Conclusions: LS-sarcotoxin and LS-stomoxyn are suitable as lead candidates for the development of novel antibiotics; however, their pharmacokinetic properties need to be improved for systemic administration.

Introduction

The spread of antibiotic-resistant bacterial pathogens is a major challenge, with some strains now showing the worrying ability to overcome even antibiotics of last resort.[1–4] The lack of antibiotics with novel targets or modes of action means that the development pipeline is insufficient to address MDR pathogens such as carbapenem-resistant *Acinetobacter baumannii* (CRAB), *Pseudomonas aeruginosa* (CRPA) and Enterobacteriaceae (CRE).[5,6] Alternative treatment options include antibodies, probiotics and bacteriophages,[7] but antimicrobial peptides (AMPs) are especially promising due to their potent antimicrobial activity and their ability to neutralize toxins.[8,9]

Insects produce the largest and most diverse repertoire of AMPs,[10] and many insect AMPs have been assembled as a library of synthetic peptides.[11–14] We previously described 23 AMPs from the medicinal maggots of the common green bottle fly *Lucilia sericata*, which colonizes habitats with remarkable microbial loads, such as carrion and infected wounds, and we tested these peptides against *Escherichia coli* and *A. baumannii*.[13,15]

Here we report the characterization of LS-sarcotoxin and LS-stomoxyn, two linear cationic AMPs from *L. sericata*, to assess their suitability as leads for systemic application in humans. Our experiments included *in vitro* and *in vivo* absorption, distribution, metabolism, excretion and toxicity (ADMET) analysis and activity profiling

Table 1. Properties of the synthetic *L. sericata* AMPs

AMP[a]	Sequence	Size	MW[b]	p*I*[b]	Charge[b]	G[c]
LS-sarcotoxin	GWLKKIGKKIERVGQHTRDATIQTIGVAQQAANVAATLK-NH_2	39	4199.86	11.63	+6.1	−0.321
all-D LS-sarcotoxin	D-(GWLKKIGKKIERVGQHTRDATIQTIGVAQQAANVAATLK)-NH_2	39	4199.86	11.63	+6.1	−0.321
LS-stomoxyn	GFRKRFNKLVKKVKHTIKETANVSKDVAIVAGSGVAVGAAM-NH_2	41	4326.13	11.72	+8.1	0.059
all-D LS-stomoxyn	D-(GFRKRFNKLVKKVKHTIKETANVSKDVAIVAGSGVAVGAAM)-NH_2	41	4326.13	11.72	+8.1	0.059

[a]'*all*-D' signifies L-amino acids were replaced by the corresponding D-amino acids.
[b]The AMP properties molecular weight (MW), isoelectric point (p*I*) and net charge at pH 7 (Charge) were calculated using software provided at http://pepcalc.com/.
[c]G, GRAVY score, total hydropathy values of all the amino acids divided by the size.[96]

under physiological conditions against an extended panel of clinical MDR Gram-negative bacteria.

Materials and methods

Ethics

All procedures involving experimentation on animal subjects have been performed according to directive 63/2010 of the European Commission implemented in German animal welfare legislation and are registered by the competent authority (district government in Darmstadt, Hesse, Germany) under FH-1008. All procedures were in accordance with policies of Sanofi on the protection of animals and the responsible use of animals in research and production.[16,17]

Antimicrobial peptides

LS-sarcotoxin and LS-stomoxyn and the corresponding D-enantiomers were produced by solid-phase synthesis at ≥90% purity (GenScript, USA). The amino acid sequences and calculated physicochemical properties are listed in Table 1.

Bacterial isolates and culture conditions

The bacterial isolates (Tables S1 and S2, available as Supplementary data at *JAC* Online) were predominantly cultured in CAMHB (Becton Dickinson, Germany). Clinical isolates were provided by the Robert Koch Institute (Wernigerode, Germany), originating from hospitalized patients in Germany (Table S2).

Inhibition of microbial growth

MIC values were determined using initial cell populations and preparation methods appropriate for each test species (Supplementary Methods). MIC_{50} and MIC_{90} values (concentrations achieving no bacterial growth in 50%/90% of the tested isolates) were determined against a panel of MDR clinical isolates as previously defined.[18]

Chequerboard assay

Interactions between the peptides and colistin were investigated using the chequerboard assay,[19] which we adapted to 384-well microtitre plates with an assay volume of 20 μL. The fractional inhibitory concentration (FIC) values for each AMP and colistin were calculated for each combination using the equations $FIC_{AMP} = C_{AMP}/MIC_{AMP}$ and $FIC_{CST} = C_{CST}/MIC_{CST}$, where MIC_{AMP} and MIC_{CST} are the MICs of LS-sarcotoxin/LS-stomoxyn and colistin, respectively, and C_{AMP} and C_{CST} are the concentrations of LS-sarcotoxin/LS-stomoxyn and colistin in combination, respectively. Fractional inhibitory concentration index (FICI) values for each combination were calculated as follows: $FICI = FIC_{AMP} + FIC_{CST}$. Values ≤0.5 indicated synergy and values >4 indicated antagonism.[20]

Serial-passage mutagenesis

Serial-passage mutagenesis tests with *E. coli* and *P. aeruginosa* were conducted by a modification of the procedure described previously.[21] Briefly, 10-fold-concentrated 1:2 dilution series of the peptides were prepared in 384-well microtitre plates over a concentration range of 5210–0.16 μg/mL in a volume of 50 μL, and 2 μL aliquots were transferred to new 384-well microtitre plates. These plates were sealed and stored at −80°C until further use. On each assay day 18 μL of the desired bacterial suspension (5×10^5 cfu/mL) was added. After incubation for 23 h, the content of the wells containing the second highest peptide concentration allowing bacterial growth was diluted 1:10000 in fresh CAMHB, and 18 μL aliquots were added to new plates containing the 10-fold concentrated peptide dilution series. This passaging was repeated for 30 days consecutively.

Time–kill kinetics

LS-sarcotoxin and LS-stomoxyn were added at 1×, 2×, 4× and 8× MIC.[22] At time points 0, 0.5, 1, 2, 4 and 5 h after inoculation, 100 μL samples were diluted 1:10 in a 7-fold dilution series in PBS (pH 7.4) and plated on CAMHB agar using an Eddy Jet 2 (IUL, Spain). After overnight incubation at 37°C, the cultures were visualized using a Flash & Go camera (IUL) to determine the cfu/mL.

Haemolysis, cytotoxicity and potassium channel interaction assays

Haemolytic activity was assessed as previously described.[23] Cytotoxicity was determined by measuring intracellular ATP levels of mycoplasma-free HepG2 cells (ATCC) using the CellTiter-Glo ATP monitoring kit (Promega).[24] NOEC (no observed effect concentration) values were recorded, describing the highest peptide concentrations at which no cytotoxic effect (cell viability >80%) or precipitation of the test item was observed. AMPs were tested at concentrations of 1.56, 3.13, 6.25, 12.5, 25, 50, 100, 200 and 400 μM. AMP interaction with the human Ether-à-go-go-Related Gene (hERG) potassium channel was analysed using an automated patch-clamp method as previously described,[25] with AMPs at concentrations of 0.12, 0.37, 1.1, 3.3, 10 and 30 μM.

Metabolic stability and plasma clearance

The metabolic stability of the AMPs was determined by measuring the half-life in human hepatocytes and calculating the human extraction ratio based on intrinsic, scaled and predicted hepatic clearance (Supplementary Methods).[26] Scaled intrinsic hepatic clearance in humans was calculated based on a weight of 25.71 g liver/kg and a hepatocellularity of

Table 2. MIC values of LS-sarcotoxin against a panel of Gram-negative clinical isolates

Species and resistance phenotype[a] (number of isolates)	$MIC_{50/90}$ (mg/L)	MIC of LS-sarcotoxin (mg/L)[b]							
		2	4	8	16	32	64	128	>128
E. coli (26)	4/8		22	4					
CST^R MEM^R (1)			1						
CST^R (9)			8	1					
MEM^R (4)			3	1					
S (12)			10	2					
E. cloacae (23)	8/8	1	9	12	1				
CST^R MEM^R (1)				1					
CST^R (3)		1		2					
MEM^R (10)			4	5	1				
S (9)			5	4					
Enterobacter aerogenes (1)			1						
CST^R MEM^R (1)			1						
K. pneumoniae (21)	4/8		12	8	1				
CST^R MEM^R (6)			4	2					
CST^R (9)			4	4	1				
MEM^R (2)			2						
S (4)			2	2					
Klebsiella oxytoca (2)			2						
MEM^R (2)			2						
S. enterica (10)	4/8		5	5					
CST^R (2)			1	1					
S (8)			4	4					
C. freundii (1)				1					
MEM^R (1)				1					
A. baumannii (20)	4/8		15	5					
CST^R MEM^R (3)			2	1					
MEM^R (16)			12	4					
S (1)			1						
Acinetobacter pittii (1)				1					
S (1)				1					
P. aeruginosa (2)								2	
MEM^R (2)								2	
S. maltophilia (2)								1	1
CST^R MEM^R (2)								1	1
M. morganii (4)									4
CST^R MEM^R (1)									1
CST^R (3)									3
S. fonticola (1)									1
CST^R MEM^R (1)									1

[a]CST^R, resistant to colistin; MEM^R, resistant to meropenem; S, susceptible to colistin and meropenem.
[b]The numbers of isolates for which the MIC value was determined are tabulated.

99×10^6 cells/g liver. Scaled predicted hepatic clearance as well as the human extraction ratio were calculated based on a hepatic blood flow of 1.24 L/h/kg.[27] Plasma clearance was determined as previously described.[28]

Pharmacological off-target profiling

Pharmacological off-target profiling was conducted by CEREP (Celle-Lévescault, France) to investigate inhibitory effects against the targets specified in Figure S1. All tests were conducted at a peptide concentration of 10 μM (42.0 and 43.3 mg/L for LS-sarcotoxin and LS-stomoxyn, respectively).

Handling of experimental mice

Six test-naive, healthy, 11-month-old, 34 ± 4 g male mice (RjOrl:SWISS, Janvier, France) were separated into two groups after delivery and kept in specific pathogen-free (SPF) facilities (open polycarbonate cages, EU TYP III, 820 cm^2) on small animal litter composed of spruce granules (LIGNOCEL FS 14, cubic granulate, 3.5–4.5 mm). The housings were enriched with chew sticks (SAFE Block, SAFE, France), paper-based nesting material (SAFE crinklets, SAFE) and a mouse house/igloo. Each group of three animals was used as an experimental group in the study without any randomization. The mice were kept at 20–24°C, 45%–65% relative

humidity and a light/dark cycle of 12 h/12 h in group housings and supplied with tap water and V1534-10 mm pellets *ad libitum*.

Acute toxicity and mouse pharmacokinetics

The peptides were resolved in Ampura® water (Fresenius, Germany) as stock solutions of 2.02±0.02 mg/g and each was administered as a single intravenous dose of 0.19±0.01 g through the tail vein to reach a final concentration of 10 mg/kg per mouse. Group 1 was treated with LS-sarcotoxin on 9 February 2017 [mouse (M) 1, 8:48:45 a.m.; M2, 11:07:48 a.m.; M3, 9:07:29 a.m.]. Group 2 was treated with LS-stomoxyn on 2 March 2017 (M1, 8:15:42 a.m.; M2, 8:19:23 a.m.; M3, 8:22:42 a.m.). No control groups were used. All procedures were performed in laboratory H826-304 (Industriepark Höchst). After administration, the mice were routinely evaluated for signs of toxicity. At 0.08, 0.25, 0.5, 1, 2, 4, 8 and 24 h after administration, 10 μL blood samples were transferred to heparin-coated tubes and then into ethanol containing 0.5% (v/v) ammonia and the plasma proteins were precipitated for 20 min at 1735 **g**. Supernatants were separated and analysed in triplicate by LC–MS/MS (Supplementary Methods) to evaluate the stability of the parent peptides. Three mice per group were used to calculate the standard deviation and the coefficient of variation in pharmacokinetic studies. After the experiment, the mice were euthanized with CO_2.

Results

Antimicrobial activity against reference strains

LS-sarcotoxin and LS-stomoxyn lacked significant activity against Gram-positive bacteria and *Candida albicans* (MIC ≥1024 mg/L) as well as the Gram-negative bacterium *Proteus mirabilis*, which is intrinsically resistant to cationic peptides (Table S1).[29,30] However, both peptides showed strong activity (MIC 4 mg/L) against the tested Enterobacteriaceae and *A. baumannii*. Furthermore, when tested against *P. aeruginosa*, LS-stomoxyn was considerably more active (MIC 8 mg/L) than LS-sarcotoxin (MIC 64 mg/L; Table S1).

Activity against an extended panel of Gram-negative clinical isolates

Next, we tested the AMPs against 114 MDR clinical isolates, which were selected based on their resistance phenotype, including but not limited to resistance to colistin and meropenem (Table S2). LS-stomoxyn was further tested against 52 isolates of *P. aeruginosa*. LS-sarcotoxin was active in the range 2–16 mg/L against isolates of *E. coli*, *Enterobacter* spp., *Klebsiella* spp., *Salmonella enterica*, *Citrobacter freundii* and *Acinetobacter* spp., resulting in MIC_{50} and MIC_{90} values of 4 and 8 mg/L, respectively (Table 2). The MIC profile of LS-stomoxyn ranged from 2 to >128 mg/L, with MIC_{50} and MIC_{90} values of 4 and 8 mg/L, respectively, except against *K. pneumoniae* (MIC_{90} 32 mg/L). The activity of LS-stomoxyn differed strikingly from that of LS-sarcotoxin by the comparably high activity against *P. aeruginosa*, with MIC values in the range 4–64 mg/L and MIC_{50}, respectively, and MIC_{90} values of 8 and 32 mg/L (Table 3). Both peptides were inactive or only weakly active (MIC ≥64 mg/L) against *Stenotrophomonas maltophilia*, *Morganella morganii* and *Serratia fonticola*, in accordance with previous findings, demonstrating that these species are intrinsically resistant to cationic peptides.[29,30]

To investigate potential correlation between colistin and/or meropenem resistance and a low susceptibility to the AMPs, we compared the signed-rank median MIC values of the two AMPs against the different resistance phenotypes (resistance to colistin and/or meropenem as well as isolates susceptible to both antibiotics) (Figure S2). No significant differences in AMP activity were observed against resistant and susceptible isolates, although the activity of LS-stomoxyn against the colistin-resistant but meropenem-susceptible strains was less pronounced (Figure S2).

Activity of all-D amino acid AMPs

To determine whether the AMPs recognize a specific target in a stereospecific manner or interact non-specifically with the lipid bilayer,[31–35] we tested the natural *all*-L and corresponding *all*-D enantiomers of both AMPs against several Gram-negative reference strains and clinical isolates (Table 4). The *all*-D LS-sarcotoxin was virtually inactive against most strains, with MIC values ≥1024 mg/L. Some residual activity (MIC 128–256 mg/L) was observed against *E. coli* RKI 131/08, *A. baumannii* ATCC 19606 and *A. baumannii* RKI 19/09. The *all*-D LS-stomoxyn displayed a clear but less pronounced decline in activity, with MIC values of 16–64 mg/L.

Activity under physiological conditions

Physiological conditions were approximated by supplementing the CAMHB medium with 150 mM NaCl or 1.25 mM $CaCl_2$. A salt-dependent increase in MIC values was no higher than 2-fold in all experiments and there was little impact on the activity of LS-sarcotoxin (Table 5). The addition of 10% (v/v) human serum boosted the antibacterial activity (Table 5). The MIC values of LS-sarcotoxin decreased by 8- to 32-fold, to ∼0.25 mg/L for *E. coli*, *K. pneumoniae* and *A. baumannii*, whereas the MIC values for LS-stomoxyn decreased by 16- to 128-fold, to ∼0.063 mg/L. In contrast, the activity against *P. aeruginosa* declined by 2- to 4-fold. To replicate physiological conditions more accurately, we tested the peptides in CAMHB adjusted to 150 mM NaCl and also supplemented with 10% (v/v) human serum, which enhanced the activity of both AMPs even further (Table 5). The MIC values of LS-sarcotoxin against *E. coli* and *A. baumannii* were 128-fold lower (0.031 mg/L) and against *K. pneumoniae* and colistin-resistant *E. coli* they were 16- to 32-fold lower (0.13–1 mg/L). The MIC values of LS-stomoxyn were ∼0.016 mg/L for *E. coli*, *K. pneumoniae* and *A. baumannii*, reflecting at least a 256-fold increase in activity. A remarkable but less pronounced 64-fold increase in activity was also observed for the colistin-resistant *E. coli* and *K. pneumoniae* isolates. The addition of mouse serum rather than human serum achieved similar although somewhat less pronounced effects on the MIC values (Table S3).

Antibacterial activity in combination with colistin

In preliminary experiments we observed 2-fold lower MIC values for LS-sarcotoxin and LS-stomoxyn when tested in the presence of sub-MIC concentrations of colistin (0.075 mg/L). For more detailed analysis, we performed chequerboard titration experiments (Figure 1). All experiments revealed a synergistic interaction between LS-sarcotoxin and colistin (FICI ≤0.5). No interaction was observed between LS-stomoxyn and colistin (FICI 1–0.6).

Development of resistance to L. sericata AMPs

Serial passaging of *E. coli* and *P. aeruginosa* for 30 days in the presence of LS-sarcotoxin or LS-stomoxyn did not result in the emergence of any resistant mutants (Figure S3).

Table 3. MIC values of LS-stomoxyn against a panel of Gram-negative clinical isolates

Species and resistance phenotype[a] (number of isolates)	$MIC_{50/90}$ (mg/L)	MIC of LS-stomoxyn (mg/L)[b]							
		2	4	8	16	32	64	128	>128
E. coli (26)	4/8		15	9	1			1	
CST^R MEM^R (1)			1						
CST^R (9)			5	3				1	
MEM^R (4)			2	2					
S (12)			7	4	1				
E. cloacae (23)	4/8	1	14	6	2				
CST^R MEM^R (1)				1					
CST^R (3)		1	1	1					
MEM^R (10)			6	3	1				
S (9)			7	1	1				
E. aerogenes (1)			1						
CST^R MEM^R (1)			1						
K. pneumoniae (21)	8/32		6	9	2	2		1	1
CST^R MEM^R (6)			3	2	1				
CST^R (9)				4	1	2		1	1
MEM^R (2)			2						
S (4)			1	3					
K. oxytoca (2)			2						
MEM^R (2)			2						
S. enterica (10)	4/8		6	4					
CST^R (2)			2						
S (8)			4	4					
C. freundii (1)				1					
MEM^R (1)				1					
A. baumannii (20)	4/8		10	9	1				
CST^R MEM^R (3)			1	2					
MEM^R (16)			9	6	1				
S (1)				1					
A. pittii (1)				1					
S (1)				1					
P. aeruginosa (54)	8/32		3	28	14	8	1		
CST^R MEM^R (1)						1			
MEM^R (51)			3	26	14	7	1		
S (2)				2					
S. maltophilia (2)							1		1
CST^R MEM^R (2)							1		1
M. morganii (4)							2		2
CST^R MEM^R (1)									1
CST^R (3)							2		1
S. fonticola (1)							1		
CST^R MEM^R (1)							1		

[a]CST^R, resistant to colistin; MEM^R, resistant to meropenem; S, susceptible to colistin and meropenem.
[b]The numbers of isolates for which the MIC value was determined are tabulated.

Time–kill kinetics

LS-sarcotoxin at 2× and 3× the MIC led to a rapid ≥3 log reduction in cfu/mL after incubation for 1 h (Figure 2). At a concentration corresponding to 8 × MIC, a ≥6 log reduction in cfu/mL was achieved after incubation for only 30 min. At 1 × MIC, LS-sarcotoxin reduced cfu/mL by 2.6 log after 1 h and did not achieve bactericidal activity. LS-stomoxyn was strongly bactericidal at all the concentrations we tested and caused a ≥3 log reduction after incubation for 1 h (Figure 2).

In vitro toxicity studies

For LS-sarcotoxin, haemolysis was observed at 1024 mg/L, whereas *all*-D LS-sarcotoxin, LS-stomoxyn and *all*-D LS-stomoxyn showed

Table 4. Activity of *all*-D enantiomers of *L. sericata* AMPs compared with the native parental *all*-L enantiomers

		MIC (mg/L)[a]			
Test strain	Phenotype	LS-sarcotoxin	*all*-D LS-sarcotoxin[b]	LS-stomoxyn	*all*-D LS-stomoxyn[b]
E. coli	S	4	1024	4	16
E. coli	MEM^R	4	128	4	16
K. pneumoniae	S	4	>1024	4	16
K. pneumoniae	MEM^R	4	>1024	4	16
A. baumannii	S	4	256	4	64
A. baumannii	MEM^R	4	128	4	32
P. aeruginosa	S	64	>1024	8	32
P. aeruginosa	MEM^R	128	>1024	8	32
E. coli	CST^R	4	>1024	4	16
K. pneumoniae	CST^R	16	>1024	8	32

[a]MIC values were determined in CAMHB for the colistin/meropenem-susceptible (S) strains *E. coli* ATCC 25922, *K. pneumoniae* DSM 30104, *A. baumannii* ATCC 19606, *P. aeruginosa* ATCC 27853, the meropenem-resistant (MEM^R) strains *E. coli* RKI 131/03, *K. pneumoniae* RKI 93/10, *A. baumannii* RKI 19/09, *P. aeruginosa* RKI 93/12, and the colistin-resistant (CST^R) strains *E. coli* RKI 6A-6 and *K. pneumoniae* RKI 19/16.
[b]Enantiomer in which L-amino acids were replaced by the corresponding D-amino acids.

Table 5. Relative increase in activity under different approximated physiological conditions

		Fold decrease in MIC compared with CAMHB[a]							
		LS-sarcotoxin				LS-stomoxyn			
Test strain[b]	Phenotype	N	C	S	S + N	N	C	S	S + N
E. coli	S	1	1	16	128	1	1	64	256
E. coli	MEM^R	1	1	32	128	1	0.5	128	256
K. pneumoniae	S	1	1	32	32	0.5	0.5	128	256
K. pneumoniae	MEM^R	0.5	1	8	32	0.5	0.5	64	256
A. baumannii	S	1	1	16	128	0.5	0.5	32	256
A. baumannii	MEM^R	1	1	16	128	1	0.5	64	256
P. aeruginosa	S	ND	ND	0.5	0.25	0.5	0.5	4	4
P. aeruginosa	MEM^R	ND	ND	0.25	0.25	1	0.5	0.5	0.5
E. coli	CST^R	ND	ND	16	32	ND	ND	32	64
K. pneumoniae	CST^R	ND	ND	8	16	ND	ND	16	64

ND, not determined.
[a]MICs were determined in CAMHB, CAMHB adjusted to 150 mM NaCl (N) or 1.25 mM $CaCl_2$ (C), CAMHB supplemented with 10% human serum (S) and CAMHB supplemented with 10% human serum and adjusted to 150 mM NaCl (S + N). Fold changes in MIC values were calculated with respect to the MIC values obtained in CAMHB.
[b]MIC values were determined for the colistin/meropenem-susceptible (S) strains *E. coli* ATCC 25922, *K. pneumoniae* DSM 30104, *A. baumannii* ATCC 19606, *P. aeruginosa* ATCC 27853, the meropenem-resistant (MEM^R) strains *E. coli* RKI 131/03, *K. pneumoniae* RKI 93/10, *A. baumannii* RKI 19/09, *P. aeruginosa* RKI 93/12 and the colistin-resistant (CST^R) strains *E. coli* RKI 6A-6 and *K. pneumoniae* RKI 19/16.

no haemolytic activity up to 1024 mg/L (Table 6). All four peptides showed minimal HepG2 toxicity, with NOECs of 100 μM (420 and 433 mg/L for LS-sarcotoxin and LS-stomoxyn, respectively; Table 6). Furthermore, as a model for cardiotoxicity, we investigated the effects of LS-sarcotoxin and LS-stomoxyn on the hERG potassium ion channel. No target-specific activity was observed at concentrations of up to 30 μM (126 and 130 mg/L, respectively), representing the highest test concentrations (Table 6).

In vitro stability studies

LS-sarcotoxin and *all*-D LS-sarcotoxin were stable, with half-lives >1000 min in human hepatocytes and calculated human extraction ratios <14% (Table 6). LS-stomoxyn was less stable, with a half-life of 77 min and a calculated human extraction ratio of 68.8%. In contrast, *all*-D LS-stomoxyn was stable, with a half-life of 4110 min and a calculated human extraction ratio of 3.9%. All four peptides were considered unstable in human, mouse and rat

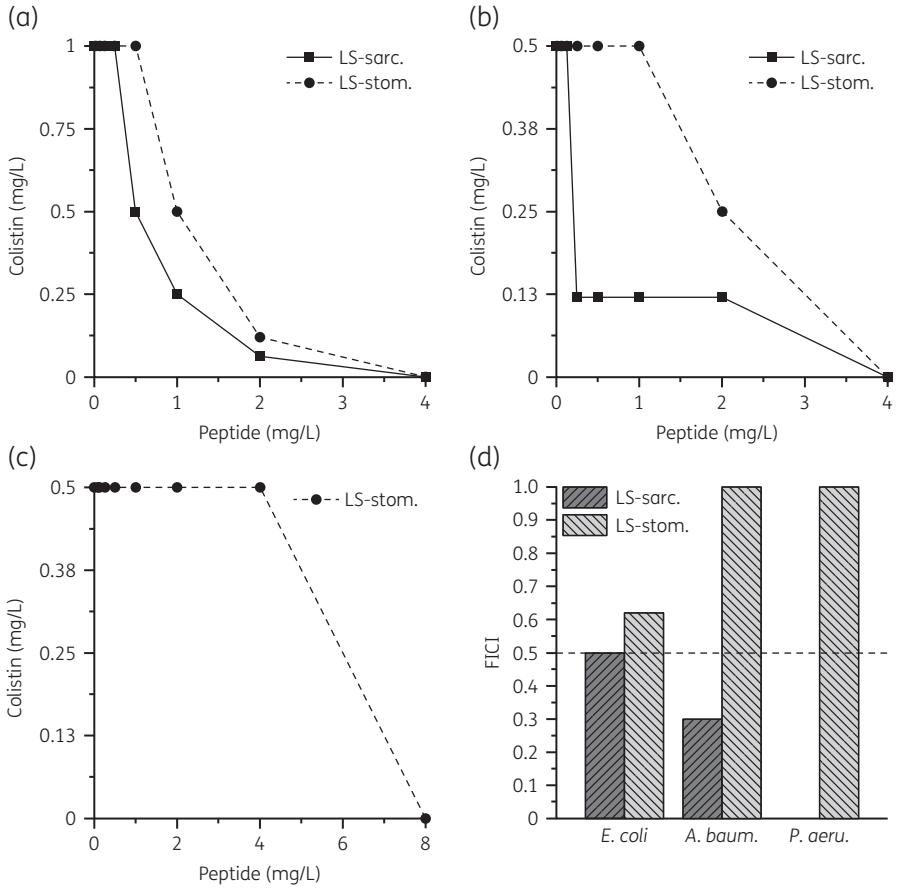

Figure 1. Interaction of LS-sarcotoxin (LS-sarc.) and LS-stomoxyn (LS-stom.) with colistin tested on *E. coli* ATCC 25922 (a), *A. baumannii* ATCC 19606 (b) and *P. aeruginosa* ATCC 27853 (c). The data points of the isobolograms represent the concentrations of the two individual compounds in different combinations leading to complete bacterial growth inhibition. The calculated FICIs are displayed in (d). Values ≤0.5 indicate synergy.

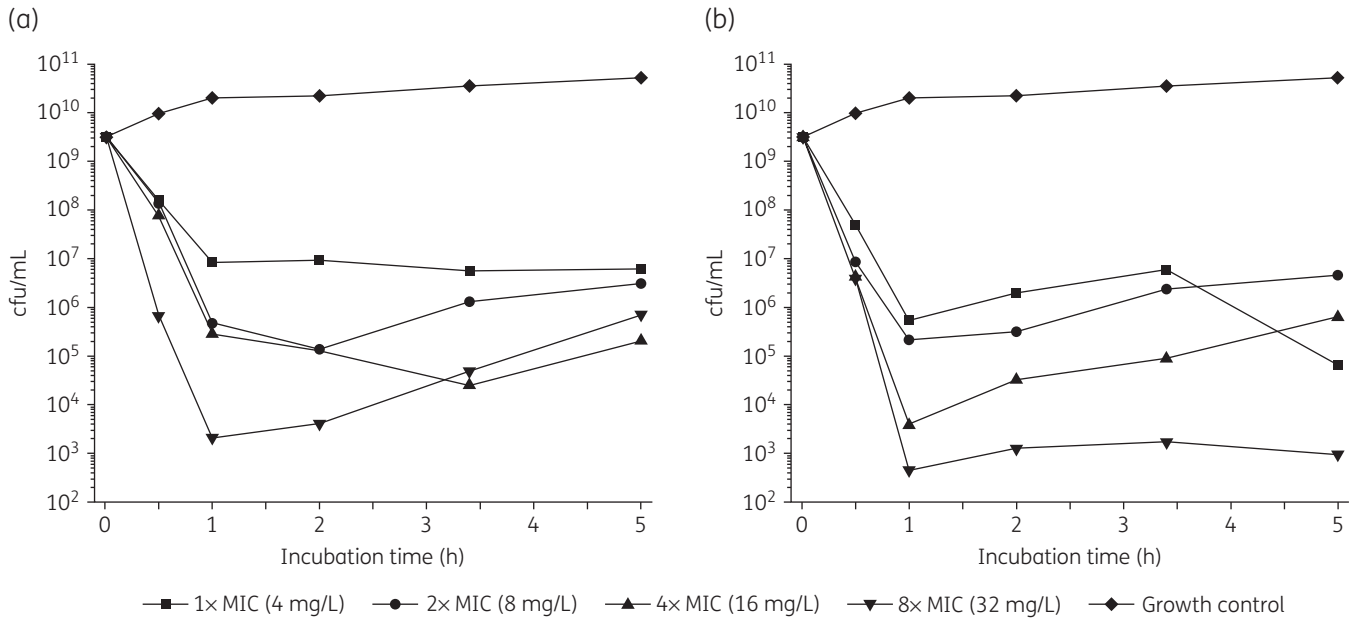

Figure 2. Kill kinetics of LS-sarcotoxin (a) and LS-stomoxyn (b) against *E. coli* ATCC 25922 determined in CAMHB. The cfu/mL values are shown according to the incubation time for different peptide concentrations.

Table 6. *In vitro* toxicity and metabolic stability of the *L. sericata* peptides

AMP	MHC[a] (mg/L)	NOEC[b] (mg/L)	IC_{50} hERG[c] (mg/L)	$t_{1/2}$ hepatocytes[d] (min)	Eh[e] (%)
LS-sarcotoxin	1024	420	>126	1060	13.9
all-D LS-sarcotoxin	>1024	420	ND	>5000	<3
LS-stomoxyn	>1024	433	>130	77	68.8
all-D LS-stomoxyn	>1024	433	ND	4110	3.9

ND, not determined.
[a]Minimal haemolytic concentration determined for human erythrocytes.
[b]Highest peptide concentration at which no cytotoxic effect (cell viability >80%) was observed for HepG2 cells.
[c]Concentration at which the hERG channel was inhibited by 50%.
[d]Half-life of AMPs determined in human cryopreserved hepatocytes.
[e]Human extraction ratio: proportion of the compound that is eliminated by one passage through the liver.

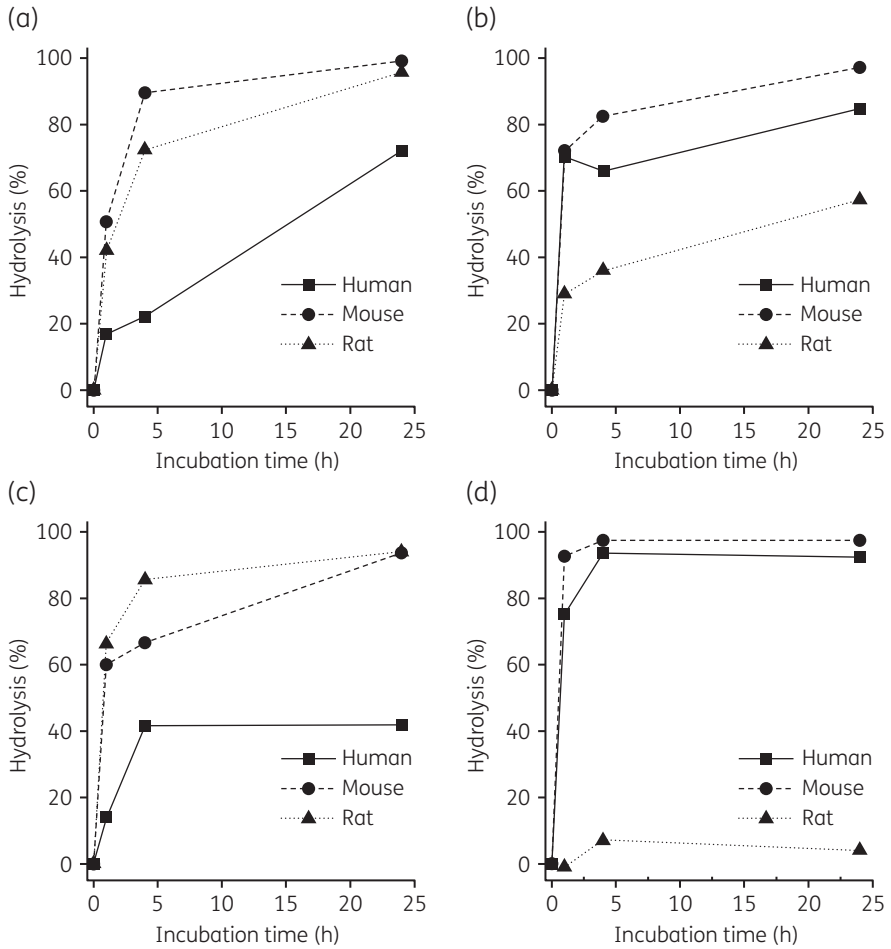

Figure 3. Stability of LS-sarcotoxin (a), *all*-D LS-sarcotoxin (b), LS-stomoxyn (c) and *all*-D LS-stomoxyn (d) in plasma from different species. The extent of hydrolytic degradation is shown according to the incubation time in human, mouse and rat plasma.

plasma after incubation for 24 h (Figure 3). Only *all*-D LS-stomoxyn was stable in rat plasma (4% hydrolysis). LS-sarcotoxin and its *all*-D enantiomer were stable in human plasma for 1 h with only 17% and 14% loss, respectively.

Pharmacological off-target profiling

Pharmacological broad-spectrum off-target profiling of LS-sarcotoxin and LS-stomoxyn against 19 G-protein-coupled receptors (GPCRs), 8 ion channels, 2 transporters and 4 enzymes

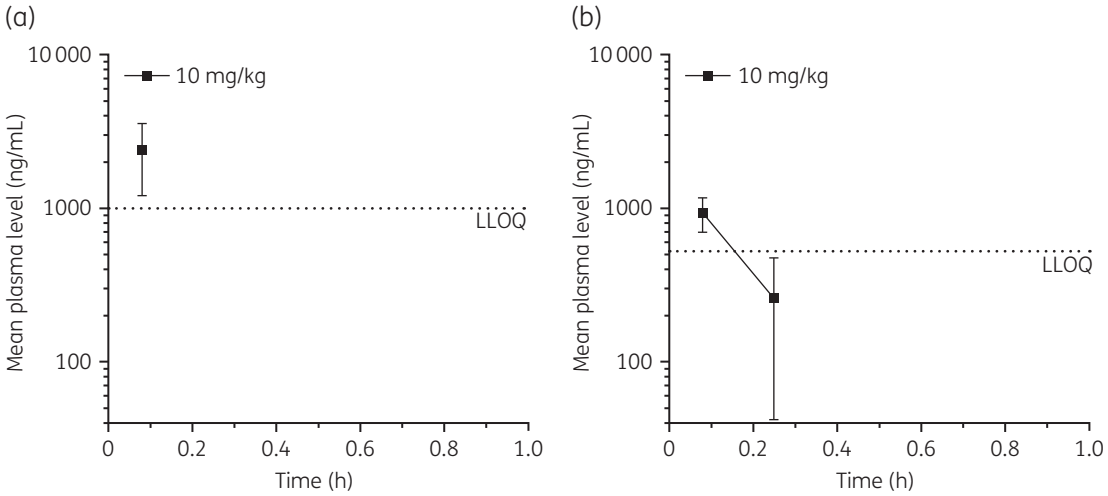

Figure 4. Mean plasma levels (±SD) of LS-sarcotoxin (a) and LS-stomoxyn (b) after a single intravenous administration of 10 mg/kg to male Swiss mice. For LS-sarcotoxin and LS-stomoxyn, the LLOQ was determined as 1000 and 250 ng/mL, respectively.

revealed few conspicuous interactions at a concentration of 10 μM (42.0 and 43.3 mg/L for LS-sarcotoxin and LS-stomoxyn, respectively) (Figure S1). We observed antagonistic inhibition of two human muscarinic acetylcholine receptors (M1 and M3) by LS-stomoxyn, reducing their activity by 49% and 44%, respectively, and antagonistic inhibition of the human M3 receptor by LS-sarcotoxin, reducing its activity by 31%.

Tolerability and mouse pharmacokinetic profiling

LS-sarcotoxin and LS-stomoxyn were each administered to 3/6 test-naive, healthy male Swiss mice at a single intravenous dose of 10 mg/kg. All mice survived without signs of toxicity or adverse events. Both peptides were rapidly cleared from the plasma. For LS-sarcotoxin, plasma concentrations above the lower limit of quantification (LLOQ) were only detected 5 min after administration, the first sampling timepoint (Figure 4a). For LS-stomoxyn, plasma concentrations above the LLOQ were detected up to 15 min after administration (Figure 4b).

Discussion

The selective activity of LS-sarcotoxin and LS-stomoxyn against Gram-negative bacteria was verified against a large panel of MDR clinical isolates, indicating the absence of cross-resistance to β-lactams, aminoglycosides, ciprofloxacin, chloramphenicol and sulfamerazine/trimethoprim. Selective activity against Gram-negative bacteria has been reported for other insect-derived AMPs[36,37] and artificial AMPs.[38] Among current clinical antibiotics, only colistin (polymyxin E) and polymyxin B display a similar activity profile,[39] highlighting the reportedly similar mechanism of polymyxins and cationic α-helical AMPs.[8,40–44]

We also observed results that highlighted differences between the *L. sericata* AMPs and polymyxins. For example, the AMPs retained full (LS-sarcotoxin) or only moderately reduced (LS-stomoxyn) activity against colistin-resistant isolates of *E. coli*, *Enterobacter cloacae*, *K. pneumoniae* and *A. baumannii* (Figure S2). Furthermore, the synergistic activity of LS-sarcotoxin with colistin would not be observable if both compounds had an identical target. Cationic AMPs from various sources are active against bacterial isolates with acquired colistin resistance, including AMPs isolated from frog skin,[45] cecropin A/melittin hybrid peptides,[46] the artificial peptides WLBU2 and WR12[47] and star-shaped peptide polymers composed of randomly polymerized lysine and valine residues, known as structurally nano-engineered antimicrobial peptide polymers (SNAPPs).[48] Other cationic AMPs, including human cathelicidin LL-37,[47] the insect cecropins A and B,[49] the porcine AMP cecropin PI[49] and the artificial tetra-branched peptide SET-M33L,[38] show cross-resistance to colistin. Whereas *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 rapidly develop resistance (>100-fold change in MIC) to colistin in serial passaging experiments,[50,51] we recovered no mutants resistant to LS-sarcotoxin or LS-stomoxyn during a 30 day passaging experiment at sub-MIC concentrations, also supporting a mode of action distinct from colistin.

Evidence for LS-sarcotoxin and LS-stomoxyn interacting with specific chiral targets rather than inducing detergent-like membrane lysis was provided by the low activity of their D-enantiomers.[34,35,52] Complete loss of activity was previously observed for the *all*-D analogue of apidaecin, a proline-rich AMP from honeybees, which was recently shown to bind specifically to the bacterial heat shock protein DnaK and to block the assembly of the 50S ribosomal subunit in a stereospecific manner.[53,54] In contrast, the L and D forms of the α-helical AMPs cecropin A, cecropin B and magainin-2 showed nearly identical antibacterial activity,[31,52,55,56] indicating that the formation of pores in lipid bilayers is sufficient.[31–35,56]

AMPs with a large hydrophobic surface area or a high cationic charge can be toxic to human cells,[57,58] but neither LS-sarcotoxin nor LS-stomoxyn showed evidence of haemolytic or cytotoxic effects. AMPs are typically less active in the presence of human serum due to salt-mediated charge repulsion, proteolytic degradation or interactions with plasma proteins.[59–61] However, rather than the anticipated increase in MIC values, we observed a reduction for both peptides in the presence of human serum, as reported previously for polymyxin B, polymyxin B nonapeptide, colistin, magainin 2 and the synthetic polymyxin derivative SPR741, which may reflect interactions with the complement system.[62–72]

However, in preliminary experiments we also observed an increase in activity when we used serum in which the complement system was inactivated by heat treatment.

Despite the increasing number of AMP candidates,[38,51,73,74] toxicity and unfavourable ADMET properties[75,76] have thus far prevented the development of AMPs for systemic application.[9,74,77] We found that LS-sarcotoxin and LS-stomoxyn were stable in the presence of hepatocytes ($t_{1/2}$ 1060 and 77 min, respectively). Chemical analysis revealed the rapid loss of peptides exposed to human, mouse or rat plasma, but there was no difference in loss rate for the L and D forms, suggesting the AMPs are binding to plasma proteins such as albumin, apolipoproteins or glycoproteins.[78–81] Although plasma protein binding of antibiotics affects pharmacological parameters,[75,82–86] this does not rule out the suitability of the peptides for systemic application given that several approved antibiotics show >90% plasma protein binding.[64,67,68,85,87] In contrast to other reported AMPs,[88–90] LS-sarcotoxin and LS-stomoxyn presented inconspicuous off-target profiles. Mouse tolerability studies revealed that LS-stomoxyn or LS-stomoxyn was well tolerated, indeed better tolerated than colistin,[38,51,91] which is approved for the clinic.[92–94] Although both peptides were rapidly cleared from mouse plasma and *in vitro* stability studies showed similar results for rat and human plasma, several strategies to increase the half-life of AMPs have been described,[38,48,60,95] which could be used to improve the observed pharmacokinetic profiles.

In conclusion, both LS-sarcotoxin and LS-stomoxyn are promising leads for the development of new antibiotics with activity against MDR Gram-negative bacteria and possibly a novel mode of action. However, the pharmacokinetic properties of the native insect AMPs need to be improved before proceeding to *in vivo* infection models.

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Transparency declarations

None to declare.

Supplementary data

Supplementary Methods, Tables S1 to S3 and Figures S1 to S3 appear as Supplementary data at *JAC* Online.

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