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In Vitro Potential of Lycosin-I as an Alternative Antimicrobial Drug for Treatment of Multidrug-Resistant *Acinetobacter baumannii* Infections

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The resistance of multidrug-resistant *Acinetobacter baumannii* (MDRAB) isolates to most traditional antibiotics results in huge challenges for infection therapy. We investigated the *in vitro* activities of both L- and D-lycosin-I against MDRAB. These two compounds displayed high antibacterial activities and rapid bactericidal effects against MDRAB. Moreover, the compounds retained their activity even at high salt (Mg^{2+} or Ca^{2+}) concentrations. These results demonstrate the potential of lycosin-I to be developed as a new antibiotic.

Acinetobacter baumannii is one of the predominant pathogens associated with nosocomial infections (1–3). The abuse of antibiotics over the last 2 decades has led to the continuous emergence of multidrug-resistant *A. baumannii* (MDRAB) isolates (4, 5). The clinical severity of infections with MDRAB isolates with intrinsic and acquired resistance has been exacerbated by the limited number of effective antibiotics. Although polymyxins and, possibly, tigecycline are considered to be the last resort of reliable treatments (6–8), the emergence of MDRAB resistance to these two types of antibiotics has been reported worldwide (9–11), which has created a pressing need to discover effective new alternative agents.

Antimicrobial peptides (AMPs) are recognized as innate immune compounds that can be extracted from insects, bacteria, animals, and plants (12–17). AMPs that possess broad-spectrum activity against bacteria and a low risk of resistance acquisition have brought new hope for overcoming microbial drug resistance (18).

In a previous study, we isolated an AMP named lycosin-I from the venom of the spider *Lycosa singoriensis* (19). The rapid inhibition of various standard strains of bacteria and fungi by lycosin-I was observed (20), but its effectiveness for the treatment of clinical isolates, particularly multidrug-resistant microorganisms, remains unexplored. In this study, we investigate the *in vitro* antibacterial properties of two isomers of lycosin-I, namely, wild-type lycosin-I (L-lycosin-I) and an unnatural lycosin-I isomer (D-lycosin-I), against MDRAB clinical isolates.

Unique *A. baumannii* isolates were collected from the Second Xiangya Hospital during the period of January to July 2013. The identification and analysis of the antibiotic susceptibilities of these strains was performed using a BD Phoenix-100 automated microbiology system (Diagnostic Systems, Sparks, MD) and an API 20 NE system (bioMérieux, Inc.). Without molecular identification, we must acknowledge that some isolates of the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex which were not *Acinetobacter baumannii* could not be excluded in our research. The results were interpreted according to the breakpoints suggested by the Clinical and Laboratory Standards Institute (CLSI) (21). The *A. baumannii* strains which were resistant to various kinds of agents, especially carbapenem antibiotics, were classified as MDRAB strains (22, 23), and the strains that were sensitive to most of the conventional clinical antibiotics were regarded as drug-susceptible isolates.

The presence of a series of genes was determined using PCR with specific primers (see Table S1 in the supplemental material). All PCR assays were performed using Red Load *Taq* master (Jena Bioscience, Jena, Germany) in a Techne thermocycler (Techne, United Kingdom). The higher prevalence of these genes in MDRAB isolates (see Table S2) further confirms the multidrug resistance of the MDRAB strains at the genetic level.

Three AMPs were used: the L isomer of lycosin-I (L-lycosin-I), the D isomer of lycosin-I (D-lycosin-I), which consists of L- and D-amino acid residues (RKGWFKAMKSIAKFIAKEKLKEHL), and a scrambled lycosin-I (S-lycosin-I), which was synthesized from the N terminus to the C terminus of lycosin-I (LHEKLKEK AIFKAISKMAKFWGKR). The peptides were synthesized and purified as described in our previous study (19).

MICs of lycosin-I. The MICs of the three isomers of lycosin-I and several other clinical drugs were determined through the broth microdilution method in accordance with the CLSI protocol (Table 1; see also Table S2 in the supplemental material) (21). With MICs ranging from 8 to 32 μg/ml, L- and D-lycosin-I exhibited more potent inhibitory activities against both drug-susceptible *A. baumannii* and MDRAB isolates than most of the traditional drugs tested, except polymyxin B, which was reported to be of high toxicity (24, 25). However, no distinct differences were observed between L- and D-lycosin-I. The MICs of S-lycosin-I ranged from 128 to >256 μg/ml, which indicates that this compound exhibits only slight activity against the microorganisms tested. There were no distinct differences between the MIC ranges

Received 11 May 2014 Returned for modification 20 July 2014
Accepted 3 September 2014
Published ahead of print 8 September 2014
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Supplemental material for this article may be found at http://dx.doi.org/10.1128/AAC.03279-14.

doi:10.1128/AAC.03279-14

TABLE 1 MICs of three types of lycosin-I and various traditional antibiotics against *Acinetobacter baumannii*

Drug[a]	MIC (μg/ml) for: MDRAB isolates (n = 18) Range	50%	90%	Drug-susceptible *A. baumannii* isolates (n = 15) Range	50%	90%
L-Lycosin-I	8–32	8	16	8–32	8	16
D-Lycosin-I	8–32	8	16	8–32	8	16
S-Lycosin-I	128–>256	256	>256	128–>256	256	>256
AMK	>256	>256	>256	4–16	8	16
SCF	32–>256	64	>256	4–16	8	16
SAM	32–>256	256	>256	4–16	8	16
IPM	32–256	64	256	1–4	2	4
MEM	32–>256	64	128	1–4	2	4
MIN	4–32	4	32	1–4	1	4
CIP	>256	>256	>256	1	1	1
PMB	1–8	2	8	0.125–1	0.25	1
TGC	4–32	4	16	0.125–1	0.25	1

[a] AMK, amikacin; SCF, cefoperazone-sulbactam; SAM, ampicillin-sulbactam; IPM, imipenem; MEM, meropenem; MIN, minocycline; CIP, ciprofloxacin; PMB, polymyxin B; TGC, tigecycline.

of L- and D-lycosin-I against MDRAB and drug-susceptible isolates.

Time-kill kinetics of lycosin-I. The time-kill curves for two types of lycosin-I (L-and D-) were determined against one representative isolate each of MDRAB and drug-susceptible *A. baumannii*, respectively, at a concentration equal to 4× MIC. Bacteria from an overnight culture were diluted with LB broth in flasks to a bacterial density of approximately 5×10^8 CFU/ml and cultured to the exponential phase. Viable colonies (CFU/ml) were counted 0, 5, 10, 20, 30, 40, 50, and 60 min after antibiotic addition through serial dilution using sterile saline and plating of 0.01-ml amounts of the serial dilutions onto LB agar. As shown by the results in Fig. 1, both L- and D-lycosin-I displayed rapid bactericidal activity against both multidrug-resistant and drug-susceptible isolates at a concentration equal to 4× MIC. L- and D-lycosin-I reduced the numbers of CFU by approximately 50% during a 30-min exposure period and by 100% within 50 min. In comparison, S-lycosin-I exhibited no obvious bactericidal activity.

Salt tolerance of lycosin-I. To determine the effects of $MgCl_2$ and $CaCl_2$ on the antibacterial activities of the two types of lycosin-I (L and D), the MICs of lycosin-I against one representative isolate each of MDRAB and drug-susceptible *A. baumannii* were measured in the presence or absence of 5 mM Mg^{2+} and Ca^{2+}, and the growth inhibition curves were plotted. The results are displayed in Fig. 2. The MICs of L- and D-lycosin-I against MDRAB and drug-susceptible strains were 4- and 2-fold higher after exposure to 5 mM Ca^{2+} and Mg^{2+}, respectively. Our results indicate that a high concentration of Ca^{2+} or Mg^{2+} reduces the antibacterial activity of these two types of lycosin-I and that Ca^{2+} exerted a more suppressive effect. However, it is worth noting that, despite their slightly reduced inhibitory activities, L- and D-lycosin-I retained their potent ability to inhibit the growth of the tested isolates in the presence of 5 mM Mg^{2+} and Ca^{2+}, with MICs of 16 μg/ml and 32 μg/ml, respectively. There were no significant differences in salt sensitivity between L- and D-lycosin-I.

It is clear that the membrane permeabilization mechanism is the dominant mechanism through which AMPs kill bacteria (18, 20). This mechanism of action is not highly specific toward a protein target, which indicates that it may escape the mechanisms involved in multidrug resistance (26). The higher efficiency of lycosin-I against MDRAB compared to that of traditional drugs makes it a prospective candidate for overcoming multidrug resistance. We compared the *in vitro* activities of L- and D-lycosin-I against MDRAB but found no significant differences in their MICs and time-kill kinetics, confirming the hypothesis that the antimicrobial activity of AMPs is not mediated by a chirality-dependent interaction with the membrane (27). The fact that lycosin-I exerts its activity against bacteria by acting on a surface target rather than interacting with a chiral center may be one of the reasons for the low risk associated with the acquisition of resistance to this compound. S-Lycosin-I displays very low inhibitory activity and no bactericidal effects on our tested isolates, even at high concentrations, which indicates that the amino acid sequence of the peptide plays a vital role in its inhibitory activity against bacteria, particularly through its binding to the membranes of target cells.

Human body fluids with high salt concentrations can deactivate AMPs. Thus, we must consider the salt sensitivity of these two types of lycosin-I, which may lead to decreases in their activity *in vivo* (28, 29). Our results demonstrated that L- and D-lycosin-I

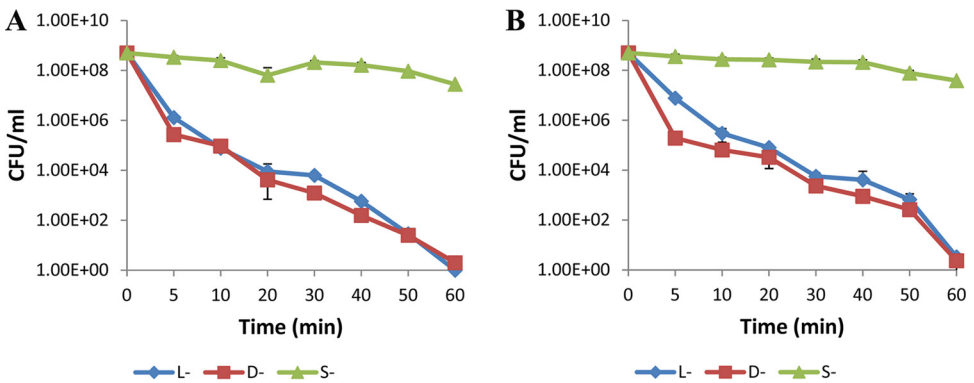

FIG 1 Time-kill curves for representative *Acinetobacter baumannii* isolates exposed to three types of lycosin-I (4× MIC). (A) Results for MDRAB isolate (isolate 9) exposed to three types of lycosin-I (inoculum, 5×10^8 CFU/ml). (B) Results for drug-susceptible isolate (isolate 25) exposed to three types of lycosin-I (inoculum, 5×10^8 CFU/ml). All of the data are expressed as the means of three independent experiments ± standard errors.

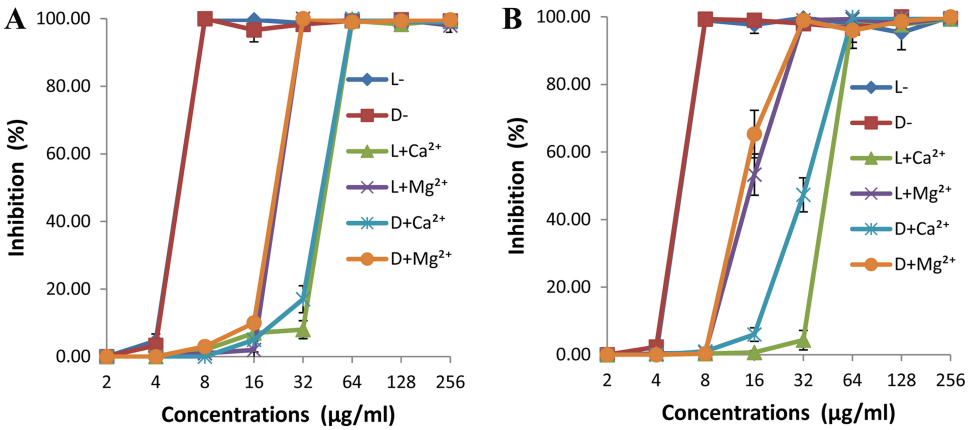

FIG 2 Effects of Mg^{2+} and Ca^{2+} on the antimicrobial activities of L- and D-lycosin-I against representative *Acinetobacter baumannii* isolates. (A) Results for MDRAB isolate (isolate 9). (B) Results for drug-susceptible isolate (isolate 25). All of the data are expressed as the means of three independent experiments ± standard errors.

would retain their activity at high salt concentrations (5 mM Mg^{2+} or Ca^{2+}), which may indicate that they can be utilized *in vivo*.

Currently, there are limited choices that are effective in the clinical treatment of MDRAB infections. The *in vitro* activities of L- and D-lycosin-I against MDRAB strains were found to be higher than those of the traditional antibiotics tested in our study. These compounds were demonstrated to have potential for the development of novel antibiotics, which offers new hope for overcoming microbial drug resistance.

ACKNOWLEDGMENTS

This research was supported by the Science and Technology Planning Project of Hunan Province, China (grant no. 2013SK2015), and the Excellent Youth Foundation of Hunan Province, China (grant 14JJ1018).

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