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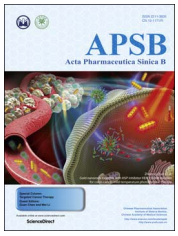

ORIGINAL ARTICLE

Identification of anti-Gram-negative bacteria agents targeting the interaction between ribosomal proteins L12 and L10

Weiwei Wang[a], Chao Liu[a], Ningyu Zhu[a], Yuan Lin[b], Jiandong Jiang[a,b], Yanchang Wang[a,c], Yan Li[a,*], Shuyi Si[a,*]

[a]*Beijing Key Laboratory of Antimicrobial Agents, Institute of Medicinal Biotechnology, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing 100050, China*
[b]*State Key Laboratory of Bioactive Substances and Function of Natural Medicine, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050, China*
[c]*Department of Biomedical Sciences, College of Medicine, Florida State University, Tallahassee, FL 32306, USA*

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Abstract Gram-negative bacteria have become the main pathogens and cause serious clinical problems with increased morbidity and mortality. However, the slow discovery of new antimicrobial agents is unable to meet the need for the treatment of bacterial infections caused by drug-resistant strains. The interaction of L12 and L10 is essential for ribosomal function and protein synthesis. In this study, a yeast two-hybrid system was established to successfully detect the interaction between L12 and L10 proteins from gram-negative bacteria *Escherichia coli*, which allows us to screen compounds that specifically disrupt this interaction. With this system, we identified two compounds IMB-84 and IMB-87 that block L12−L10 interaction and show bactericidal activity against *E. coli*. We used glutathione-*S*-transferase (GST) pull-down and surface plasmon resonance (SPR) assays to demonstrate that these compounds disrupt L12−L10 interaction *in vitro* and the target of compounds was further confirmed by the overexpression of target proteins. Moreover, protein synthesis and elongation factor G-dependent GTPase activities are inhibited by two compounds. Therefore, we have identified two antibacterial agents that disrupt L12−L10 interaction by using yeast two-hybrid system.

*Corresponding authors.
E-mail addresses: 13391513480@163.com (Yan. Li), sisyimb@hotmail.com (Shuyi. Si).

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1. Introduction

Gram-negative bacteria have become the main pathogens in clinic, and one main reason is the generation of drug-resistant bacterial strains[1–4]. Unfortunately, very few drugs are available in clinical treatment of drug-resistant gram negative strains. Also, few new agents are under development to keep pace with the emerging of drug-resistant bacteria[5–7]. Therefore, it is imminent to identify new antimicrobial agents with novel targets to deal with the emergence of drug-resistant bacteria.

Proteins carry out all cellular functions in each organism and the ribosome is the factory where protein synthesis occurs. The difference between ribosome structures in bacteria and human cells allows some antibiotics to kill bacteria specifically[8]. For example, gentamicin binds to the A site of 16S ribosomal RNA to prevent protein synthesis in bacteria[9]. Tetracycline inhibits protein synthesis in bacteria because it prevents aminoacyl-tRNA from entering the A site during translation[10]. Now, protein–protein interaction inhibitors provide new opportunities for drug discovery. The ribosome is a multi-protein complex and the protein–protein interactions in a ribosome are attractive targets for new antibiotics because of their central role in protein synthesis and cellular functions.

In bacteria, ribosomal proteins L12 (encoded by the *rplL* gene) and L10 (encoded by the *rplJ* gene) are part of the stalk, which belongs to the large ribosomal subunit (50S). It has been shown that the elongation factors EF-G and EF-Tu are recruited to the stalk by the L12 C-terminal domain to enhance the GTPase activity[11,12]. Consistently, a ribosomal stalk lacking L12 is unable to interact with elongation factors[13]. The C-terminal α-helix of L10 anchors two or three L12 dimers by associating with the N-terminal domains of L12, and disruption of L12–L10 interaction prevents the binding of EF-G and EF-Tu to the stalk and causes the loss of ribosomal GTPase activity[14]. A yeast two-hybrid system has been established to identify small molecules that block the interaction between L12 and L10 proteins from *Mycobacterium tuberculosis*. L12–L10 interaction appears to be a potential target for anti-tuberculosis agents[15,16]. In addition, L12–L10 interaction is highly conserved in Gram-negative bacteria, but these two proteins show low homology with corresponding human proteins. Therefore, the L12 − L10 interaction could be used to screen new agents that kill Gram-negative bacteria.

In this study, we developed a screening system based on the interaction between *Escherichia coli* ribosomal proteins L12 and L10. Firstly, L12–L10 interaction is confirmed by yeast two-hybrid (Y2H) system, with which we can identify the compounds that specifically inhibit this interaction. After screening, two compounds IMB-84 and IMB-87 were selected. These compounds block L12–L10 interaction and inhibit the growth of *E. coli* with certain toxicity to mammalian cells. Surface plasmon resonance (SPR) and glutathione-*S*-transferase (GST) pull-down were further used to confirm that IMB-84 and IMB-87 inhibit L12–L10 interaction *in vitro*. Consistently, both EF-G-dependent GTPase activity and ribosome-mediated protein synthesis are inhibited by these two compounds in vitro. In addition, overexpression of either L12 or L10 in *E. coli* increases the minimum inhibitory concentration (MIC) of these two compounds, indicating that L12 and L10 are likely the targets *in vivo*. In summary, using the Y2H assay, we successfully identified new antibacterial agents targeting the L12–L10 interaction in Gram-negative bacteria.

2. Materials and methods

2.1. Regents

The yeast two-hybrid system was purchased from Clontech (Dalian, China). The expression vectors pET-16b, pET-30a and pGEX-4T-1 were obtained from Novagen (Shanghai, China). The compound library is a combination of synthetic (from Enamine Ltd., Ukraine and the Institute of Medicinal Biotechnology, CAMS, China). All the compounds were suspended in DMSO and stored at 4 °C.The purity of compounds is higher than 90%. Monoclonal antibodies (anti-HA, anti-c-Myc and anti-His) were supplied by Cwbio (Beijing, China). Glutathione sepharose 4B was purchased from GE Healthcare (Uppsala, Sweden). T4 DNA ligase, restriction endonucleases and DNA polymerase were purchased from TaKaRa (Dalian, China). All other chemicals were from Sigma. The small molecule synthesized by MedPharma Partners (Boston, MA, USA). Compounds IMB-84 and IMB-87 were synthesized by the Institute of Medicinal Biotechnology and were suspended in DMSO at 10 mg/mL concentration. Further dilutions for use in the SPR assays and cell culture were done in buffer or culture medium.

2.2. Plasmid construction

The DNA fragments of *rplJ* and *rplL* genes that encode L10 and L12 proteins, respectively, were amplified by PCR from *E. coli* ATCC 25922 genomic DNA. The primer pairs were designed as follows: *rplJ* forward primer, 5′-CTCATATGGCTTTAAATCTTCAAGAC-3′, *rplJ* reverse primer, 5′-ATGGATCCTTAAGCAGCTTCTTT-3′; *rplL* forward primer, 5′-CTCATATGTCTATCACTAAAGAT-CAAAT-3′, *rplL* reverse primer, 5′-ATGGATCCTTATTTAACTT-CAACTT-3′. After digestion with *Nde*I and *Bam*HI, the PCR fragments were inserted into pGADT7 (DNA activation domain, AD) to generate plasmids pAD-L10 and pAD-L12, in such way that the DNA fragments of L10 and L12 were fused in frame with Gal4 transcription activating domain. Similarly, the DNA fragments for L10 and L12 proteins were inserted into pGBKT7 (DNA binding domain, BD) to generate pBD-L10 and pBD-L12, in which the L10 and L12 fragments were fused in frame with Gal4 DNA binding domain. The control plasmids pAD-T, pBD-53 and pBD-lam were obtained from Clontech.

For the construction of the His-fusion plasmids, the gene for L10 protein was amplified by PCR with primer pairs: forward primer, 5′-GGAATTCCATATGGCTTTAAATCTTCAAGAC-3′, reverse primer, 5′-CGCGGATCCAAAGCAGCTTCTTTCGCAT-3′. After digestion with *Nde*I and *Bam*HI, the fragment was inserted into pET30a vector; the resulting recombinant plasmid pET30a-L10 will express L10 protein with 6 × His-tag at the C terminal. After digestion with *Nde*I and *Bam*HI, the DNA fragment for L12 protein from pAD-L12 was ligated with pET16b to generate recombinant plasmid pET16b-L12, which has 6 × His-tag at the N terminal of L12 protein.

For the construction of the GST-fusion plasmids, the gene for L12 protein was amplified by PCR with primer pairs: forward primer, 5′-CGCGGATCCATGTCTATCACTAAAGATCAAATCA-3′, reverse primer, 5′-CCGCTCGAGTTTAACTTCAACTTCAGCGCCAGCTTCT-3′. This PCR fragment was inserted into the pGEX-4T-1 expression vector after digestion with the *Bam*HI and *Xho*I. All constructs were sequenced for verification.

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