Mechanism of action of a macrodiolide antibiotic produced by actinomycetes

Aoi Kimishima
Ōmura Satoshi Memorial Institute, Kitasato University

Research objective

Various *Clostridioides difficile* infections, including antimicrobial-associated enterocolitis and pseudomembranous colitis, have become a worldwide problem.[1)] Considering this background, luminamicin, a macrodiolide compound exhibiting selective and potent antibacterial activity against *C. difficile*, was identified from the culture broth of *Streptomyces* sp. OMR-59 at the Satoshi Omura Memorial Institute.[2)] Luminamicin was shown to be superior to vancomycin in in vivo hamster infection experiments. Therefore, luminamicin is expected to have a novel mechanism of action because of its superior antibacterial spectrum and characteristic structure compared to those of existing drugs; however, this remains to be elucidated. Therefore, in this study, we identified target molecules to elucidate the mechanism of action of the anti-anaerobic activity of actinomycete luminamicin so that it can be applied to the discovery of new anti-infective drugs.

Methods

To elucidate the mechanism of action, we first obtained a luminamicin-resistant strain by subculturing the wild-type strain, *C. difficile* (ATCC BAA-1382 strain 630) with repeated treatment with luminamicin. The total DNA of each strain was extracted for point mutation analysis. The factors essential for resistance were identified by comparing strains with different degrees of resistance. Next, a chemical probe was synthesized in parallel with the analysis of point mutations in luminamicin-resistant *C. difficile*. In the investigation of the structure-activity relationship of luminamicin, the conversion to maleimide was found to be tolerable to some extent and the C21 position was very reactive and easily underwent conjugate addition. Additionally, because the activity of the conjugate adduct was lost, luminamicin was speculated to act covalently on the target molecule at position 21. As the chemical probe for luminamicin, we designed a maleic probe that could be derivatized while maintaining its activity. If, as speculated, the probe acts covalently with the target molecule at position 21, probe A reacts with the *C. difficile* lysate and the probe-target molecule complex is labeled by click chemistry with an azide and a fluorescent agent. The bound proteins are then analyzed using fluorescence as an indicator. Alternatively, if luminamicin interacts non-covalently with the target molecule, a probe containing a diazirine linker that can form a strong covalent bond can also be synthesized. A pull-down assay of the *C. difficile* lysate is then performed. Finally, putative target molecular gene variants of the *C. difficile* strains were

generated for candidates narrowed by genomic analysis and pull-down assays. As the target molecule is assumed to be essential for bacterial survival, a strain overexpressing the assumed target molecule was generated, and drug sensitivity to luminamicin was compared with that of the wild-type strain (if the putative molecule is the target, the MIC (Minimal inhibitory concentration) in the overexpressing strain is increased). Gene mutants were generated using pRPF185, a shuttle vector for *Escherichia coli* and *C. difficile*, as described by Fagan et al.[3] In this study, we identified the target molecule of luminamicin.

Results

Luminamicin exhibits selective activity against standard laboratory-adapted anaerobic bacterial strains. However, its antimicrobial activity against drug-resistant clinical isolates has not been evaluated. Therefore, we evaluated the antibacterial activity of luminamicin, luminamicin exhibited selective antibacterial activity against gram-positive obligate anaerobes. Furthermore, the antibacterial activity of luminamicin against *C. difficile* was comparable to that of vancomycin, and it was found to be a narrow-spectrum antibiotic against *C. difficile*. Because luminamicin is also effective against fidaxomicin-resistant *C. difficile* strains, it is expected to have a different mechanism of action from that of the existing drugs (vancomycin, peptidoglycan synthesis, fidaxomicin, and RNA polymerase). Subsequently, we attempted to obtain a luminamicin-resistant *C. difficile* strain. When *C. difficile* was plated on media containing luminamicin, a luminamicin-resistant *C. difficile* strain (MIC value =2–8 mg/mL) was developed. Strains with the highest MIC were selected for mutation analysis. Whole-genome analyses of the mutant and wild-type strains were carried out. No mutations were found in RNA polymerase, which is the target of the existing drug fidaxomicin. However, a partial amino acid deletion in the C-terminal variable region (CDIF 630_RS03155) of the cell wall protein CwpV was confirmed. In addition, mutations were detected in a hypothetical protein (CDIF 630_02150). These results strongly suggest that luminamicin has a mechanism of action that is different from that of existing drugs. Derivative synthesis and structure-activity relationship studies were performed to prepare chemical probes using a natural luminamicin product. First, highly electrophilic moieties of luminamicin, such as maleic anhydride and enol ethers, were derivatized because many bioactive compounds are covalently attached to the target binding site at electrophilic positions. Finally, nine derivatives were synthesized and their activities were evaluated. Consequently, maleic anhydride and enol ether moieties were found to be important functional groups for maintaining antimicrobial activity, and the 14- and 10-membered lactones may be required because of their proper molecular conformations. Noteworthily, the acyl derivatives showed no antimicrobial activity, suggesting that the C-18 OH group was an essential functional group. These findings and those of previous reports suggest that all the molecules of luminamicin are required for its anti-

anaerobic activity. Furthermore, given the high electrophilicity of the conjugated enol ethers, luminamicin may bind to target molecules via the conjugated enol ether moiety. Furthermore, the oxygen-bridged decalin backbone and 14- and 10-membered lactone moieties were required to lock luminamicin into the proper conformation. Presently, the puldane assay is performed using a maleimide compound with a synthesized propargyl group, and the selection of the candidate molecule for the target molecule is advanced.

Conclusion

Sequence analysis of luminamicin-resistant *C. difficile* indicated that CwpV (CDIF 630_03155) or a hypothetical protein (CDIF 630_02150) may be the molecular targets. Because CwpV has been proposed to be a resistance factor against phage infection, we postulated one possible hypothesis that luminamici protect against CwpV and *C. difficile* phage infection. However, because the C-terminal region of CpwV is known to vary, the possibility that this deletion occurred spontaneously during the construction of luminamicin-resistant mutants should be considered. In view of this and the unknown function of the hypothetical protein (CDIF 630_02150), further investigation is required to identify the mechanism of action of luminamicin. In addition, the derivatives were synthesized from natural products and evaluated for biological activity against 27 pathogens, including clinical isolates. Based on the results of the bioactivity tests, maleic anhydride and enol ether moieties are considered important functional groups for maintaining antibacterial activity against *C. difficile*, and 14- and 10-membered lactones may influence the adoption of proper molecular conformations.

References

1) Bartlett, J. G., Gerding, D. N. (2008), *Clin. Infect. Dis.* **15:** S12–8.
2) Ōmura, S., Iwata, R., Iwai, Y., Taga, S., Tanaka, Y., Tomoda, H. (1985), Luminamicin, A new antibiotic production, isolation and physico-chemical and biological properties. *J. Antibiot.* (Tokyo). **38:** 1322–1326.
3) Fagan, R. P., Fairweather, N. F. (2011), *Clostridium difficile* has two parallel and essential Sec secretion system. *J. Biol. Chem.* **286:** 27483–27493.