論文の内容の要旨

Abstract

Mechanistic analysis of novel therapeutically effective antimicrobial agents identified using silkworm bacterial infection model

（カイコ細菌感染モデルを用いて同定された治療効果を示す新規抗菌薬の機能解析）

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Introduction

Emergence of resistant bacterial strains shortly after the clinical use of antibiotics has made infections difficult to treat. In order to continuously overcome the infectious diseases, discovery of novel antimicrobial agents with novel mechanism of action is utmost important. Despite the need of the novel antimicrobial agents, less attention is paid by pharmaceutical industries on this field due to poor outcomes. Although many screening programs have attempted to identify antimicrobial agents, the discovery of therapeutically effective novel compounds is very difficult and has not been reported in recent years. This can be attributed to the conventional method of antibiotic discovery, *in-vitro* screening followed by *in-vivo* screening, where ethical issues make it difficult for the use of animal models at the early stage of drug development. In fact, the use of mammalian models to examine the pharmacodynamics requires larger and sophisticated space, skilled personnel; is costly and associated with ethical issues. To address these issues, I propose use of silkworm infection model for identification of novel therapeutically effective antimicrobial agents. Silkworm model not only reduces the time and cost of experiments, but also requires less space and there are no ethical issues

surrounding its use. Here, I summarize the identification of two novel therapeutically effective antimicrobial agents: kaikosin E and compound 363 by using silkworm bacterial infection model. Moreover, a detailed insight on the identification of mechanism of action of these antimicrobial agents is provided.

Results

I. Kaikosin E

a. Characterization of kaikosin E: Kaikosin E, a novel therapeutically effective antibiotic, was isolated from culture supernatant of a lysobacter species by using silkworm infection model. Kaikosin E was effective against silkworm infected with *Staphylococcus aureus* with an effective dose fifty (ED_{50}) value of 0.3 µg/g•larva. It was also effective in mouse infection model with an ED_{50} value of 0.6 mg/kg, more potent than vancomycin. The structure of kaikosin E was elucidated by MS/MS and NMR analyses as shown in **Figure 1**. Kaikosin E is a cyclic lipopeptide containing 12 amino acid residues and a short fatty acid chain with a molecular mass of 1617. It was effective against Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA) with minimum inhibitory concentration (MIC) value of 4 µg/ml and was also effective against Mycobacterium (MIC: 8 µg/ml). Kaikosin E inhibited the biosynthesis of the macromolecules DNA, RNA, protein, and peptidoglycan in exponentially growing *S. aureus*. It exerted bacteriolytic activity and showed bactericidal activity with killing of 99% of bacteria within one minute of exposure to it. It dissipated the membrane potential in *S. aureus* even at concentrations much lower than that of MIC value suggesting the potent membrane damaging effect **(Figure 2).** Mice did not die when injected with a dose of 400 mg/kg kaikosin E, i.e., the ratio of lethal dose fifty to effective dose fifty (LD_{50}/ED_{50}) was

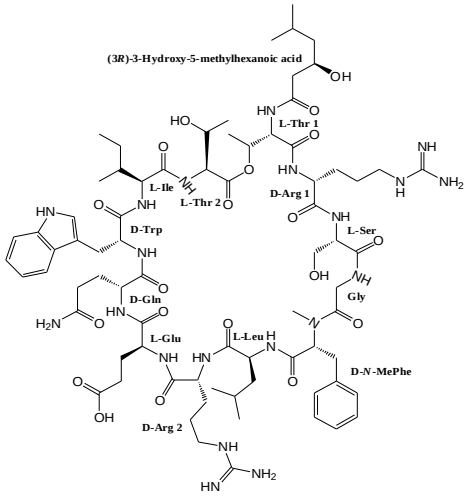

Figure 1

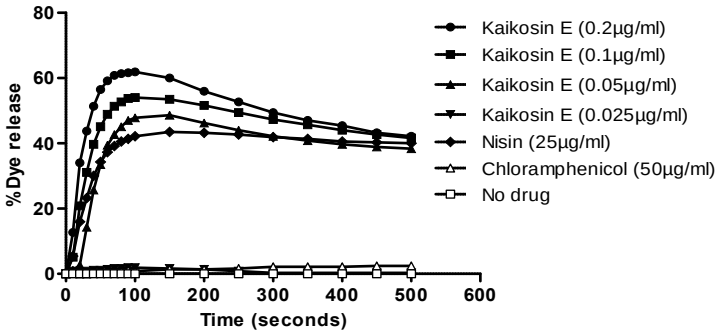

Figure 2

more than 600 indicating the wide range of therapeutic index and low toxicity. Based on its therapeutic activity and low toxicity, kaikosin E has a strong potential to be a candidate for clinical application.

b. Cellular target of kaikosin E in *S. aureus*: To reveal the cellular target of kaikosin E, I isolated mutants of *S. aureus* resistant to it. *S. aureus* strain RN4220 was treated with a mutagen ethyl methanesulfonate and cultured on agar plates with different concentrations of kaikosin E. Resistant mutants that grow at 30°C but do not grow at 43°C, referred as temperature sensitive (TS) mutants, were further selected from the strains resistant to kaikosin E. I took advantage of the fact that TS phenotype correlates with mutations in essential genes. Usually, the mutations conferred are point mutations leading to change in an amino acid sequence of a protein, the stability of protein becomes dependent upon the temperature and the protein cannot function at higher temperatures but can still

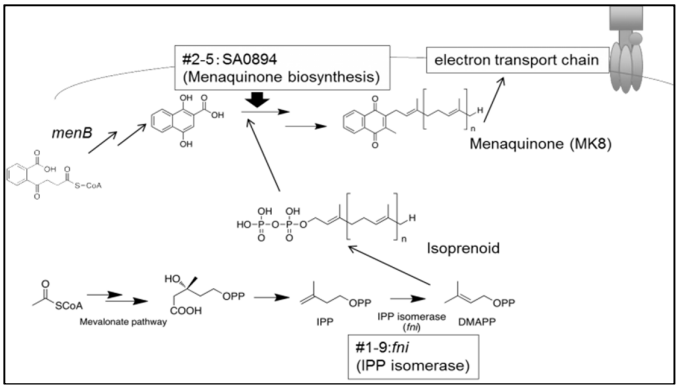

Figure 3

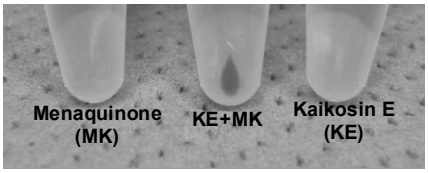

Figure 4

function at lower temperatures. This strategy helped to ignore other nonessential mutations that are less likely related to the target. I identified two TS strains resistant to kaikosin E. Analysis of these two TS strains revealed the presence of point mutations in *fni* (SA1236) and *menA* (SA0894) genes, respectively. Both of the genes are essential genes required for menaquinone biosynthesis, a key component of respiration in *S. aureus* (**Figure 3)**. Since *fni* and *menA* genes were mutated independently in two strains, wild-type *fni* or *menA* were inserted in the respective strain, which complemented the TS phenotype in each strain suggesting that these two mutations were responsible for the TS phenotype of the mutants. Mutations in these two genes in kaikosin E resistant strains and involvement of these genes in the menaquinone biosynthetic pathway led to the speculation that menaquinone might be the target of kaikosin E. This was further evident from direct binding of kaikosin E to menaquinone (**Figure 4**). Furthermore, antimicrobial activity of kaikosin E attenuated upon addition of external menaquinone. The results unequivocally mentioned that menaquinone is the cellular target of kaikosin E. Since menaquinone does not exist in mammalian cells where its function is replaced by ubiquinone, the effect of addition of external ubiquinone on the antimicrobial activity of kaikosin E was tested. No change in the activity of kaikosin E upon addition of ubiquinone further provided the evidence for selectivity of kaikosin E towards microorganisms. This is

the first report to reveal menaquinone as the target of any antimicrobial agents till date.

II. Compound 363

A novel antimicrobial agent, compound 363 was discovered from chemical library of about 100,000 compounds screened on the basis of therapeutic effect in silkworm infection model. To date, compound 363 is not known to have antimicrobial activity. It showed antibacterial activity against methicillin-susceptible *S. aureus* (MSSA) and MRSA with MIC value of 6.3 µg/ml and was bacteriostatic. It showed therapeutic activity in silkworm infection model with ED_{50} value of 39 µg/g•larva. It inhibited RNA synthesis in *S. aureus* (**Figure 5**). TS mutants resistant to compound 363 were obtained and their whole genome was sequenced by a next generation sequencer to identify mutated gene responsible for the resistance. The *sigA* gene encoding RNA polymerase sigma factor was found to have a point mutation among two of the resistant mutants. *sigA* is an essential gene responsible for initiation of transcription of house-keeping genes. The mutation in the *sigA* gene explained the result of inhibition on RNA synthesis in *S. aureus* by this compound. Phage transduction was performed to check if the mutation in *sigA* was responsible for resistance to compound 363. From the two resistant strains, 78% and 87% of the transductants were susceptible to compound 363, which is close to the calculated expected value of 85%. The whole genome sequence data suggested that no other gene close to *sigA* gene had mutations in both the strains. This, together with the phage transduction results, suggests that mutation in *sigA* gene is responsible for resistance conferred to compound 363.

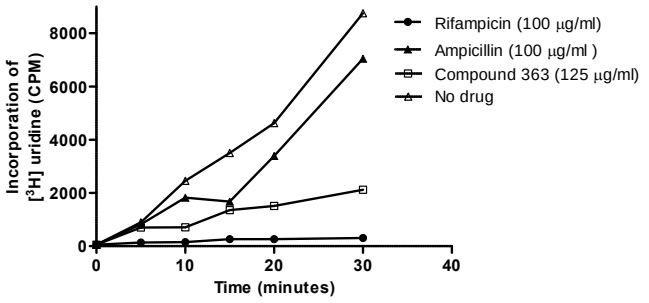

Figure 5

Discussion

Based on the silkworm infection model, two novel therapeutically effective antimicrobial agents were identified. A novel strategy of TS screening was applied to find the target of kaikosin E as menaquinone. Lack of menaquinone in mammals explained its selective toxicity towards microorganisms and showed that kaikosin E has a great potential for clinical applications. This is the first report to reveal menaquinone as a target of an antibiotic. For compound 363, mutation in *sigA* gene was found to be responsible for resistance conferred to this compound suggesting that RNA polymerase sigma factor might be the target and involved in the mechanism of antibacterial action of

compound 363. The mechanism found from this study can be further exploited to the screening, identification and design of novel therapeutically effective drug molecules ultimately providing novel insights for development of novel antimicrobial agents. These findings showed that silkworm infection model can be applied to identify novel therapeutically effective antimicrobial agents.

Publications

1. Paudel A, Hamamoto H, Kobayashi Y, Yokoshima S, Fukuyama T, and Sekimizu K. Identification of novel deoxyribofuranosyl indole antimicrobial agents. Journal of Antibiotics (Tokyo), 65:53-57 (2012).
2. Sekimizu N, Paudel A, and Hamamoto H. Animal welfare and use of silkworm as a model animal. Drug Discovery and Therapeutics, 6:226-229 (2012).