Fatty acid synthesis is a target for antibacterial activity of unsaturated fatty acids

Chang Ji Zheng[a], Jung-Sung Yoo[a], Tae-Gyu Lee[b], Hee-Young Cho[c], Young-Ho Kim[d], Won-Gon Kim[a,*]

[a] *Korea Research Institute of Bioscience and Biotechnology, P.O. Box 115, Yusong, Daejeon 305-600, Republic of Korea*
[b] *Crystalgenomics, Inc., Pungnap-dong, Songpa, Seoul 138-736, Republic of Korea*
[c] *Pharmaceutical Screening Team, Korea Research Institute of Chemical Technology, Yuseong, Daejeon 305-600, Republic of Korea*
[d] *Chungnam National University, College of Pharmacy, Daejeon 305-764, Republic of Korea*

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Abstract Long-chain unsaturated fatty acids, such as linoleic acid, show antibacterial activity and are the key ingredients of antimicrobial food additives and some antibacterial herbs. However, the precise mechanism for this antimicrobial activity remains unclear. We found that linoleic acid inhibited bacterial enoyl-acyl carrier protein reductase (FabI), an essential component of bacterial fatty acid synthesis, which has served as a promising target for antibacterial drugs. Additional unsaturated fatty acids including palmitoleic acid, oleic acid, linolenic acid, and arachidonic acid also exhibited the inhibition of FabI. However, neither the saturated form (stearic acid) nor the methyl ester of linoleic acid inhibited FabI. These FabI-inhibitory activities of various fatty acids and their derivatives very well correlated with the inhibition of fatty acid biosynthesis using [^{14}C] acetate incorporation assay, and importantly, also correlated with antibacterial activity. Furthermore, the supplementation with exogenous fatty acids reversed the antibacterial effect of linoleic acid, which showing that it target fatty acid synthesis. Our data demonstrate for the first time that the antibacterial action of unsaturated fatty acids is mediated by the inhibition of fatty acid synthesis.

Keywords: Unsaturated fatty acid; Linoleic acid; Fatty acid synthesis; Antibacterial; Enoyl-acyl carrier protein reductase

1. Introduction

The antibacterial activity of long-chain unsaturated fatty acids have been well known for many years. Fatty acids function as the key ingredients of antimicrobial food additives which inhibit the growth of unwanted microorganisms [1]. Linoleic and oleic acids are antibacterial components in the herbs (*Helichrysum pedunculatum* and *Schotia brachypetala*) used for dressing wounds during male circumcision rituals in South Africa [2,3]. Besides normal fatty acids, fatty acid derivatives showing potent antimicrobial activities exist in nature. These are mainly found in microorganisms, algae, or plants, which may mediate chemical defense against microorganisms [4–6]. Additionally, long-chain unsaturated fatty acids are bactericidal to important pathogenic microorganisms, including Methicillin-resistant *Staphylococcus aureus* [7–9], *Helicobacter pylori* [10,11], and *Mycobacteria* [12]. These antibacterial actions of fatty acids are usually attributed to long-chain unsaturated fatty acids including oleic acid, linoleic acid, and linolenic acid, while long-chain saturated fatty acids, including palmitic acid and stearic acid, are less active [7,9,10,12]. However, their primary molecular target still remains unknown.

Fatty acid synthesis (FAS) in bacteria is essential to the production of a number of lipid-containing components, including the cell membranes [13]. Bacterial fatty acid synthesis is carried out by a set of individual enzymes which are collectively known as type II, while mammalian fatty acid is mediated by a single multifunctional enzyme-acyl carrier protein (ACP) complex referred to as type I. FabI is an enoyl-ACP reductase which catalyzes the final and rate-limiting step of the chain elongation process of the type II FAS. Since there is a lack of an overall sequence homolog with the corresponding one of humans, FabI has been identified as a target for antibacterial drug development [14]. Indeed, FabI has been revealed as a target of the broad spectrum biocide, triclosan, which is used as an antibacterial additive in a wide range of consumer goods and was widely thought to be a non-specific biocide which attacks bacterial membranes [15,16]. The site of action of isoniazid, used in the treatment of tuberculosis for 50 years, was also determined to be the mycobacterial FabI homolog (termed InhA) [17].

During the course of our screening for FabI inhibitors from natural resources for new antibacterial-drug development, we frequently experienced the isolation of unsaturated fatty acids as potent inhibitors of FabI. This led us to examine whether unsaturated fatty acids are selective inhibitors of FabI and this inhibition is related to the inhibition of fatty acid synthesis and their antibacterial activity.

2. Materials and methods

2.1. Materials

Oleic acid, linoleic acid, linolenic acid, stearic acid, palimitic acid, palmitoleic acid, arachidonic acid, oleic acid methyl ester, linoleic acid methyl ester, and arachidonic acid methyl ester were purchased from Sigma. The unsaturated fatty acids are purchased all as the *cis* form. [1-^{14}C] acetate (57 μCi/μmol) and L-[U-^{14}C] leucine (306 μCi/μmol) were purchased from Amersham.

*Corresponding author. Fax: +82 42 860 4595.
E-mail address: wgkim@kribb.re.kr (W.-G. Kim).

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2.2. Synthesis of trans-2-octenoyl N-acetylcysteamine thioester (t-o-NAC-thioester)

To a solution of 2-octenoic acid (1138 mg, 8 mmol), *N*-acetylcysteamine (954 mg, 8 mmol), and 4-dimethylaminopyridine (2064 mg, 18.4 mmol) in dichloromethane at room temperature was added *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (3527 mg, 18.4 mmol). After overnight the reaction solution was concentrated under vacuum, and usual aqueous workup. The residue was purified by flash chromatography on silica gel (Hexane/EA = 3:1) to give the title compound (1363 g, 70%) as a solid.

^{1}H NMR (300 MHz, $CDCl_3$) δ 0.90 (t, J = 6.5 Hz, 3H), 1.26–1.33 (m, 4H), 1.45–1.50 (m, 2H), 1.97 (s, 3H), 2.20 (q, J = 7.2 Hz, 2H), 3.09 (t, J = 6.2 Hz, 2H), 3.46 (q, J = 6.2 Hz, 2H), 6.03 (br s, 1H), 6.07–6.15 (m, 1H), 6.89–6.99 (m, 1H).

2.3. Cloning of the fabI gene

The full length the fabI gene was amplified from genomic DNA obtained from *Escherichia coli* or *S. aureus* (ATCC 26695). After confirming the DNA sequence, the gene was cloned into a modified pET21b vector (Novagen, USA) which has 6 His-tag coding regions at the N-terminus of the insert. After transforming the ligated mixture into BL21(DE3) with ampicillin resistance, the resultant colonies were screened for their ability to overexpress proteins of the correct size after induction by IPTG. To obtain FabI, the bacteria were cultured in Luria–Broth media and induced by 0.5 mM IPTG at 18 °C for 18 h. The cells were collected by centrifugation, resuspended in a buffer A (50 mM Tris(pH 8.0) + 300 mM NaCl + 5 mM imidazole) and disrupted by microfludizer (Model M-110L, Microfluidics, USA). Soluble fraction was applied onto a Ni-NTA (Hi-trap, 5 ml, Amersham) and washed by buffer A, followed by elution in buffer A containing 500 mM imidazole. The fusion protein was further purified by Superdex 200 (Amersham) in a buffer B (50 mM Tris (pH 8.5) + 200 mM NaCl + 2 mM DTT), and the purified protein was stored at −20 °C until use.

2.4. FabI assay

Assays were carried out in half-area, 96-well microtitre plates. Compounds were evaluated in 100 μl assay mixtures containing components specific for each enzyme (see below). Reduction of the *t*-o-NAC substrate analog was measured spectrophotometrically by following the utilization of NADH or NADPH at 340 nm at 30 °C for the linear period of the assay. *S. aureus* FabI assays contained 50 mM sodium acetate, pH 6.5, 400 μM *t*-o-NAC, 200 μM NADPH, and 150 nM *S. aureus* FabI. The rate of decrease in the amount of NADPH in each reaction well was measured by a microtiter ELISA reader using SOFTmax PRO software (Molecular Devices, California, USA). The inhibitory activity was calculated by the following formula: % of inhibition = 100 × [1 − (rate in the presence of compound/rate in the untreated control)]. IC_{50} values were calculated by fitting the data to a sigmoid equation. An equal volume of dimethyl sulfoxide solvent was used for the untreated control. *E. coli* FabI assays contained 50 mM sodium phosphate, pH 7.5, 200 μM *t*-o-NAC, 200 μM NADH, and 150 nM *E. coli* FabI. The inhibitory activity was determined in the same methods as for *S. aureus* FabI, as described above.

2.5. Determination of MIC

The antibacterial activities of the test compounds were evaluated using clinically isolated bacterial strains (Hoechst, Germany). The strains were inoculated into 3 ml of Fleisch extract broth (Beef extract 1%, peptone 1%, NaCl 0.3%, $Na_2HPO_4 \cdot 12\,H_2O$ 0.2%, pH 7.4–7.5. For *Streptococcus pyogenes*, 10% horse serum was supplemented.) and cultured on a shaking incubator at 37 °C for 18 h. Test compounds were serially diluted in 2-fold dilutions from 2 mM to 15 μM. The 1.5 ml volume of each diluted solution was mixed with 13.5 ml of Muller Hinton agar (Difco, USA) and plated. The overnight-cultured strains were 100-fold diluted with broth on a 96-well plate. The diluted bacterial culture media were then inoculated (10^4 CFU/spot) on the prepared agar plates by an automatic inoculator (Dynatech, USA). The plates were incubated at 37 °C for 18 h. The lowest concentration that prevented the growth of each bacterium was determined to be MIC.

2.6. [1-^{14}C]acetate incorporation

S. aureus was grown to mid-log phase in LB medium. Each 1 ml culture was treated with drugs for 10 min. An equal volume of DMSO solvent was added to the untreated control. 2 μCi of [1-^{14}C] acetate was then added to the cultures and incubated at 37 °C for 1 h in a shaker. After being harvested by centrifugation, the cell pellets were washed twice with PBS buffer. The total cellular lipids were then extracted with chloroform–methanol–water. The incorporated radioactivity in the chloroform phase was measured by scintillation counting. Data were expressed as percentage inhibition of incorporation compared with the untreated control.

2.7. L-[U-^{14}C]leucine incorporation

S. aureus were prepared in the same manner as [1-^{14}C]*acetate incorporation*. Each 1 ml culture was treated with drugs and an equal volume of DMSO solvent as the untreated control for 10 min. 0.6 μCi of L-[U-^{14}C] leucine was then added to the cultures and incubated at 37 °C for 1 h in a shaker. The incorporation was terminated by the addition of 10% (wt/vol) trichloroacetic acid (TCA) and cooling on ice for 20 min. The precipitated material was collected on Whatman GF/C glass microfiber filters, washed with TCA and ethanol, dried, and counted in a scintillation counter.

2.8. Supplementation of exogenous fatty acids

S. aureus was grown to mid-log phase in LB medium and diluted 1000-fold in the same medium. An 100 μl of the diluted cell suspension (2×10^5 cells) were used to inoculate to each well of a 96-microtiter plate containing 95 μl of LB media with inhibitors at the concentration of MIC. 5 μl of the serially diluted fatty acid solution was supplemented, and the cell suspension was incubated at 37 °C for 18 h. The bacterial growth was determined by measuring at 650 nm using a microtiter ELISA reader. Since fatty acids are easily transported into cells from the medium as an ethanolic suspension [18], the fatty acids were added as ethanolic solutions. An equal volume of ethanol solvent was added for the untreated control.

3. Results

3.1. Inhibition of FabI by linoleic acid

The ability of linoleic acid to specifically inhibit FabI was investigated in an in vitro spectrophotometric assay using *S. aureus* FabI and the enoyl-ACP substrate analog, *t*-o-NAC-thioester. As shown in Fig. 1, the addition of increasing concentrations of linoleic acid to the reaction potently inhibited the reduction of *t*-o-NAC-thioester by NDAPH with an IC_{50} of 39 μM. We expanded our analysis to include 4 additional unsaturated fatty acids, palmitoleic acid, oleic acid, linolenic acid, and arachidonic acid, to investigate whether the inhibition of FabI was a characteristic of this class of antibacterial compound. All of the tested unsaturated fatty acids exhibited inhibition of *S. aureus* FabI with IC_{50}s between 25 and

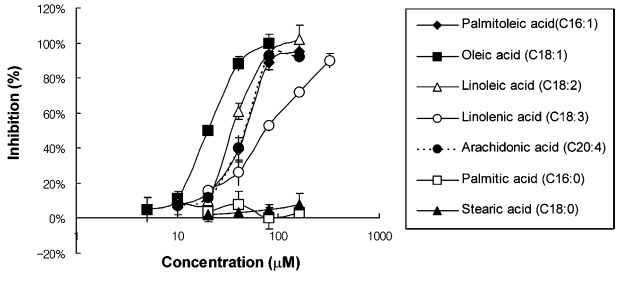

Fig. 1. Inhibitory effects of long-chain fatty acids on *S. aureus* FabI. The values were represented as the means ± S.D. in triplicates obtained from two independent experiments.