Gel-elongation assay for type II fatty acid synthesis

Jun Wang (jun_wang2@merck.com)
Merck Research Laboratories
Srinivas Kodali
Andrew Galgoci
Sheo Singh

Method Article

Keywords: FabH, FabG, FabA, FabF, FabB, FabI, platensimycin, fatty acid synthesis, Urea-PAGE, Gel-elongation, FASII

Posted Date: June 23rd, 2006

DOI: https://doi.org/10.1038/nprot.2006.134

Abstract

Introduction

Type II fatty acid synthesis \(FASII) is essential to bacterial cell viability. The significant differences between bacteria and humans in organization, structure of enzymes, and the role played by fatty acids make this pathway an attractive target for antibacterial drug discovery \(refs. 1,2). Two marketed antibacterial agents that target the FabI enzyme are triclosan \(antiseptic) and isoniazid \(an anti-Mycobacterium tuberculosis agent) \(refs 3,4). Two natural products, cerulenin \(ref. 5) and thiolactomycin \(ref. 6) which selectively inhibit the condensation enzymes FabH and FabF/B, were discovered more than two decades ago. New screening efforts and chemical modifications of existing compounds have been attempted to identify more selective and potent inhibitors. To determine the selectivity of the inhibitors identified during screening efforts we developed gel-elongation assay using crude bacterial lysate directly to determine the target specificities of fatty acid synthesis inhibitors \(refs. 7,8).

Reagents

Reagents: DTT was from Fisher \(BP172-5); beta-mercaptoethanol was from Bio-Rad \(161-0710). \[2-^{14}C]Malonyl-CoA \(60 mCi/mmol, Perkin Elmer, NEC612), ACP \(Sigma, A7303) was pretreated with 3 mM DTT on ice for 20 min, aliquoted and stored at -80 °C. Cerulenin and thiolactomycin were purchased from Sigma. Triclosan could be obtained from VWR, 003384. Urea, PVDF membrane, 10X Tris/Glycine buffer and native protein sample buffer were purchased from Bio-Rad.

Equipment

Gel electrophoresis apparatus, Phosphor Screen and PhosphorImager scanner

Procedure

1) FASII gel-elongation assay was done by preincubating 0.25 - 2 µg of _E. coli_ or _S. aureus_ lysate with a serial dilution of inhibitors at room temperature for 20 min in 50 µl of buffer containing 100 mM sodium phosphate \(pH 7.0), 5 mM EDTA, 1 mM NADPH, 1 mM NADH, 150 µM DTT, 5 mM ß-mercaptoethanol, 20 µM acetyl-CoA or n-octanoyl-CoA or any other acyl-CoA as needed, 4% DMSO, and 8 µM of ACP pretreated with DTT. 2) The reaction was initiated by addition of 10 µl of water-diluted \[C-14]malonyl-CoA, which gave a final concentration of 4 µM malonyl-CoA. 3) The reaction was incubated at 37°C for 30 min for _E. coli_ lysate and 60 min for _S. aureus_ lysate. 4) After the reaction, 10 µl \(plus 10 µl 2X native sample buffer) of each sample was directly applied to a l6% polyacrylamide gel containing a range of 0.4 M to 4 M urea as needed. 5) The gel was resolved and transfered to a polyvinylidene difluoride \(PVDF) membrane, exposed to Phosphor Screen and visualized by using a PhosphorImager scanner.

Timing

Two days. Day one to run FASII assay, resolve it on the gel and overnight transfer of protein onto PVDF membrane. Day two to expose the phosphorscreen to protein bound to the PVDF membrane and scan image using PhosphorImager scanner

Critical Steps

Urea concentration is critical for separation of chain lengths of acyl-ACPs. The activity of the lysate varies among bacterial strains. So, the required FASII activity of the lysate needs to be optimized by titration. Acetyl-CoA is not an ideal substrate for _S. aureus_ \(ref. 9).

Anticipated Results

In bacteria, FabH catalyzes the first condensation reaction using acetyl-CoA and malonyl-ACP to produce acetoacetyl-ACP. This product is reduced by FabG, dehydrated by FabA/Z to produce C4:1\(Δ2)-ACP, and then reduced second time by FabI to make butyryl-ACP \(C4:0-ACP)which is substrate for FabF/B condensing enzymes. Cerulenin, a selective FabF/B inhibitor, at 200 µg/ml accumulates butyryl-ACP and thiolactomycin \(333 µg/ml) inhibits both FabH and FabF/B condensing enzymes resulting in a minor accumulation of butyryl-ACP while leaving a majority of malonyl-ACP unreacted. Triclosan \(100 µg/ml) treatment leads to the accumulation of C4:1\(Δ2)-ACP due to its inhibition of FabI. In the gel elongation assay using _E. coli_ extracts \(Fig. 1), the inhibition observed with platensimycin \(167 µg/ml) was similar to that seen with cerulenin, and different from that observed with thiolactomycin and triclosan, indicating that platensimycin selectively targets FabF/B. Similarly, titration of platensimycin using a _S. aureus_ gel elongation assay, with octanoyl-CoA \(C8:0) and malonyl-CoA as substrates, showed an IC_{50} of 0.2 µg/ml \(Fig 2). At higher concentrations of platensimycin an accumulation of malonyl-ACP was observed, demonstrating that this inhibitor does not block FabD, a malonyl CoA:ACP transacylase. The lower concentrations of platensimycin produced partial inhibition of elongation activity and accumulations of C2n:0-ACP \(n>4), further indicating that the target of inhibition in _S. aureus_ is the FabF condensing enzyme.

References

1. Heath, R. J. & Rock, C. O. Fatty acid biosynthesis as a target for novel antibacterials. Curr. Opin. Investig. Drugs 5, 146–153 \(2004). 2. White, S. W., Zheng, J., Zhang, Y. M. & Rock, C. O. The structural biology of type II fatty acid biosynthesis. Annu. Rev. Biochem. 74, 791–831 \(2005). 3. Heath, R. J., Yu, Y.-T., Shapiro, M. A., Olson, E. & Rock, C. O. Broad Spectrum Antimicrobial Biocides Target the FabI Component of Fatty Acid Synthesis. J. Biol. Chem. 273, 30316-30320 \(1998). 4. Banerjee, A. et al. inhA, a gene encoding a target for isoniazid and ethionamide in _Mycobacterium tuberculosis_. Science 263, 227-230 \(1994). 5. Matsumae, A., Nomura, S. & Hata, T. Studies on cerulenin. IV. Biological characteristics of cerulenin. J. Antibiot. \(Tokyo) 17, 1–7 \(1964). 6. Noto, T., Miyakawa, S., Oishi, H.,

Endo, H. & Okazaki, H. Thiolactomycin, a new antibiotic. III. _In vitro_ antibacterial activity. J. Antibiot. \(Tokyo) 35, 401–410 \(1982). 7. Kodali, S. et al. Determination of selectivity and efficacy of fatty acid synthesis inhibitors. J. Biol. Chem. 280, 1669–1677 \(2005). 8. Young, K. et al. Discovery of FabH/FabF inhibitors from natural products. Antimicrob. Agents Chemother. 50, 519–526 \(2006). 9. Qiu, X. et al. Crystal structure and substrate specificity of the beta-ketoacyl-acyl carrier protein synthase III \(FabH) from _Staphylococcus aureus_. Protein Sci. 14, 2087-2094 \(2005)

Figures

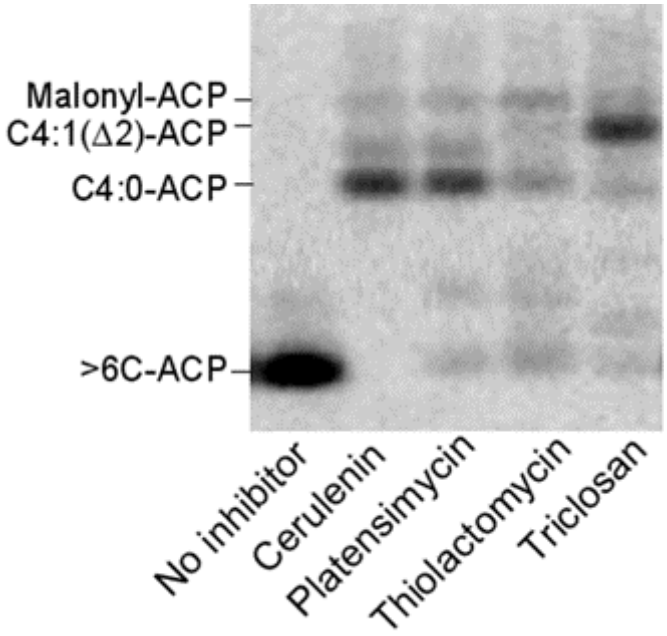

Figure 1

Determination of target selectivity _E. coli_ fatty acid synthesis (FASII) gel-elongation assay used 4 µM of [2-^14^C]malonyl-CoA (60 mCi/mmol) and 20 µM of acetyl-CoA as substrates. The reaction was carried out at 37°C for 30 min with or without an inhibitor, directly applied to a l6% polyacrylamide gel containing 0.5 M urea and was resolved using electrophoresis (0.5 M urea-PAGE). Without an inhibitor the reaction was completed and produced final products of long chain (>6C) acyl-ACPs. With addition of cerulenin (200 µg/ml) or platensimycin (167 µg/ml), the reaction was blocked showing major accumulation of butyryl-ACP (4:0-ACP). With thiolactomycin (333 µg/ml), more malonyl-ACP and less butyryl-ACP were accumulated. In the presence of triclosan (100 µg/ml), a FabI inhibitor, C4:1(Δ2)-ACP was accumulated. A weak band shown above butyryl-ACP in the presence of cerulenin or platensimycin is not defined, but is likely acetyl-ACP, an intermediate product of malonyl-ACP decarboxylation carried out by FabH.

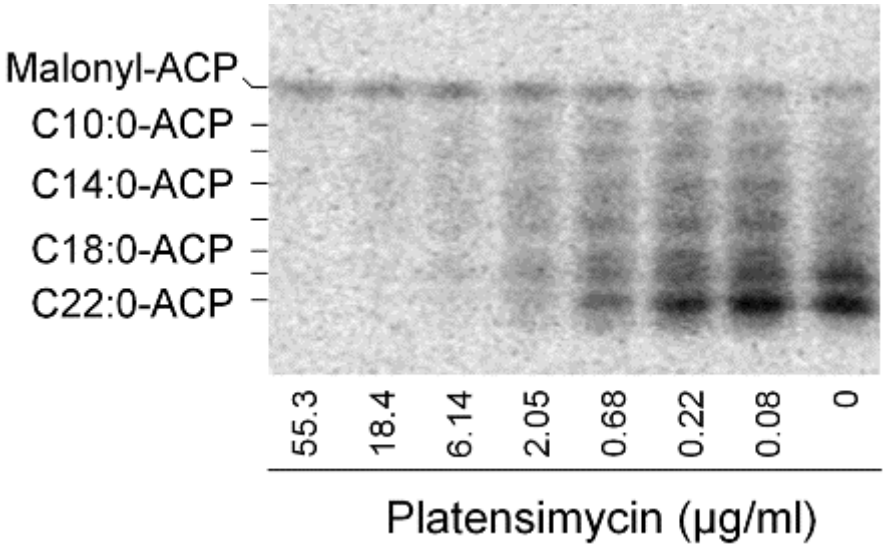

Figure 2

S. aureus FASII gel-elongation assay This assay was similar to the _E. coli_ FASII assay except that acetyl-CoA was replaced with 20 µM of octonoyl-CoA (C8:0). The reaction was carried out for 60 min with a serial dilution of platensimycin and resolved on 4 M urea-PAGE. Platensimycin inhibited _S. aureus_ FabF (saFabF) in fatty acid synthesis pathway with an IC~50~ of 0.2 µg/ml. Similar experiments were repeated at least three times with reproducible results.