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The Impact of Fatty Acids on the Antibacterial Properties of N-Thiolated β -Lactams

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Abstract

Bacterial fatty acid synthesis (FAS) is a potentially important, albeit controversial, target for antimicrobial therapy. Recent studies have suggested that the addition of exogenous fatty acids (FA) to growth media can circumvent the effects of FAS-targeting compounds on bacterial growth. Consequently, such agents may have limited *in vivo* applicability for the treatment of human disease, as free FAs are abundant within the body. Our group has previously developed N-thiolated β -lactams and found they function by interfering with FAS in select pathogenic bacteria, including MRSA. To determine if the FAS targeting activity of N-thiolated β -lactams can be abrogated by exogenous fatty acids, we performed MIC determinations for MRSA strains cultured with the fatty acids oleic acid and Tween 80. We find that, whilst the activity of the known FAS inhibitor triclosan is severely compromised by the addition of both oleic acid and Tween 80, exogenous FAs do not mitigate the antibacterial activity of N-thiolated β -lactams towards MRSA. Consequently, we propose that N-thiolated β -lactams are unique amongst FAS-inhibiting antimicrobials, as their effects are unimpeded by exogenous FAs.

Keywords

MRSA; antimicrobial activity; fatty acid biosynthesis; resistance; mechanism

Considerable interest has recently developed in targeting bacterial Type II fatty acid synthesis (FAS) for antimicrobial therapy [1]. This is derived from the fact that FAS is essential for bacterial growth, and distinct from mammalian Type I fatty acid synthesis [2]. Despite the attractiveness of this pathway as a therapeutic target, there has been controversy concerning its suitability [3–5]. A recent study demonstrated that *Streptococcus agalactiae*, a causative agent of neonatal meningitis, can survive the activity of both triclosan and cerulenin (inhibitors of FabI and FabF/B, respectively) when culture media is supplemented with long-chain fatty acids [4]. *S. aureus* has also been demonstrated to survive cerulenin activity in the presence of both short-chain and long-chain fatty acids [4]; however these claims have recently been contested in a study by Balemans et al [3]. Despite this

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controversy, major pharmaceutical companies are investigating the potential use of two FAS inhibitors, platensimycin (inhibitor of FabF) and platencin (inhibitor of FabF/H) as antimicrobial agents. Each has broad-spectrum activity against Gram-positive pathogens, including methicillin-resistant *S. aureus* (MRSA), and demonstrates low-level resistance rates of 2×10^{-8} and 1×10^{-9} , respectively [6, 7].

Our group has previously reported on N-thiolated β -lactams, a new family of synthetic β -lactams whose structure-activity profiles and antibacterial effects are distinct to all other known β -lactam antibiotics [8, 9]. These compounds possess potent bacteriostatic activity towards a select group of pathogenic bacteria, including *S. aureus* (and MRSA), *B. anthracis* and *M. luteus*; weaker activity against *N. gonorrhoeae*, *B. fragilis*, *M. tuberculosis* and *H. influenza*; and no activity at all towards many other common bacteria. This unusual spectrum of bioactivity seemingly corresponds to low intracellular levels of glutathione, and unusually high concentrations of cytosolic coenzyme A (CoA), in susceptible organisms [8]. Unlike all previously-known β -lactam antibiotics, these N-thiolated variants are non-cidal to bacteria, and do not alter cellular morphology or cell wall macrostructure. The compounds also do not possess the appropriate peripheral functionality (an ionizable side chain) needed to bind to bacterial transpeptidases, which mediate cell wall biosynthesis, or β -lactamases produced by MRSA strains, which cause hydrolysis of the β -lactam ring [8]. As such, N-thiolated β -lactams are completely unique in their mode of action, affecting type II FAS instead of cell wall biosynthesis [8]. Our earlier work suggests that these compounds induce bacteriostatic effects by sulfenylating the cysteinyl thiol of CoA to form a mixed alkyl-CoA disulfide species within the cytoplasm, which has previously been shown to cause sulfenylation of the FabH active site cysteine [10]. The observation that N-thiolated β -lactams alter FAS II pathways in MRSA and other pathogenic bacteria provides opportunities for additional investigation of drug resistance mechanisms and drug discovery.

In this report, we focus on addressing whether exogenous fatty acids in growth media can mitigate the antimicrobial activity of N-thiolated β -lactams against MRSA. Accordingly, a broadly multi-drug resistant clinical isolate, USA-100 (the leading hospital-acquired MRSA in the USA), was cultured in growth media containing the fatty acids Tween 80 and oleic acid. We also conducted these analyses in the presence of BSA (used to resuspend Tween 80 and oleic acid), and for the control compounds vancomycin, a mechanistically distinct antibiotic, and triclosan, a commercially used FAS inhibitor. When the MIC of vancomycin was tested we determined, as expected, that it remained constant and unaffected by the supplementation of growth media with fatty acids (figure 1). Conversely, the MIC of triclosan was severely impacted by the addition of fatty acids. Whilst TSB and TSB supplemented with BSA revealed an MIC of 0.02 g L^{-1} for triclosan, this value rose by 100-fold in the presence of oleic acid ($\text{MIC} = 2 \text{ g L}^{-1}$), and greater than 500-fold in the presence of Tween 80 ($\text{MIC} > 10 \text{ g L}^{-1}$). To investigate the antibiotic performance of N-thiolated β -lactams under fatty acid growth conditions, we selected our most potent anti-MRSA derivative, N-sec-butylthiolated β -lactam **1** (figure 2). When this compound was tested, we observed only a modest 2-fold increase in MIC when comparing bacterial growth in TSB ($\text{MIC} = 1 \text{ g L}^{-1}$) versus TSB supplemented with oleic acid ($\text{MIC} = 2 \text{ g L}^{-1}$). Interestingly, this increase in MIC was identical to that observed with TSB supplemented with BSA alone, suggesting the small change in MIC did not the result from addition of oleic acid, but the BSA used to resuspend it. When using Tween 80 we saw an additional, albeit again small, increase in MIC to 3-fold ($\text{MIC} = 3 \text{ g L}^{-1}$) that observed with TSB. Again, Tween 80 is resuspended in BSA, thus the fold change in MIC between TSB + BSA and TSB + Tween 80 is only 1.5-fold. This suggests that the proposed FabH-mediated inhibition of FAS by N-thiolated β -lactams is only weakly circumvented by the import of environmental fatty acids. The same cannot be said of triclosan, a known FAS inhibitor, whose activity appears to be strongly mitigated by exogenous fatty acids. It should be noted that, whilst the data

presented herein is for MRSA USA-100, we find it to be representative of similar analyses conducted with a variety of *S. aureus* strains (data not shown).

To further investigate the effects of β -lactam **1** on MRSA we performed spontaneous resistance assays. Tryptic Soy Agar plates were prepared containing $5\times$ the MIC of β -lactam **1**, and inoculated in eight separate replicates with MRSA cells, to a combined inoculum of 2.02×10^{10} cfu ml⁻¹. From this we isolated only 36 colonies, giving a spontaneous mutation frequency of 5.61×10^{-8} . MIC analysis of these mutants revealed that 35 of these strains now had MICs of 50 μ g ml⁻¹, whilst a lone isolate had resistance of 10 μ g ml⁻¹. An attempt to generate 2nd step β -lactam **1**-resistant mutants, using a $2\times$ MIC approach, was unsuccessful in yielding additionally resistant strains. Interestingly, when similar analyses were performed using MSSA isolates we found a more than 12-fold decrease in spontaneous mutation frequency, to 7.1×10^{-9} . This perhaps suggests that existing resistance mechanisms intrinsic to MRSA strains may play a role in circumnavigating the effects of this compound, as compared to MSSA isolates. Given that β -lactam **1** may induce FabH inhibition via sulfenylation of CoA, we sequenced the *fabH* gene from the wild-type and representative mutant isolates, to determine if any alterations had occurred in its coding region. Sequencing data for each strain revealed the DNA sequence of the mutants contained no differences in *fabH* sequence when compared to the parental strain.

The lack of *fabH* based mutations in β -lactam **1**-resistant MRSA mutants suggests that this protein may not readily tolerate alterations in amino sequence, due to its essential nature. This is perhaps unlikely as there are a number of amino acid alterations in FabH sequence when one compares the various sequenced genomes of *S. aureus*. One is thus led to speculate that the inhibition of FAS by β -lactam **1** may occur by alternative pathways. However, our MIC data reveals that unlike other FAS inhibitors, the addition of exogenous fatty acids only weakly abrogates the effect of this compound. Therefore, it appears that N-thiolated β -lactams are unique amongst FAS-inhibiting antimicrobials in that their activity is unimpeded by exogenous fatty acids. Given this highly unique property, and the lack of toxicity displayed by these compounds towards human cells [11], we believe that N-thiolated β -lactams may hold significant potential for the treatment of MRSA infections. Further investigations into the mechanisms of action and *in vivo* efficacy of these compounds are continuing in our laboratories.

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Figure 1. The effects of exogenous fatty acids on the antimicrobial activity of β -lactam 1
The MIC values for vancomycin, triclosan and β -lactam 1 towards MRSA were determined during growth in: TSB (white), TSB + 0.5% BSA (light grey), TSB + 0.01% oleic acid (dark grey) and TSB + 0.1% Tween 80 (black). The data presented is from at least 3 replicate experiments showing identical values.

Figure 2. N-Thiolated β -lactam 1