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Confirmation of the Antibacterial Mode of Action of SB-219383, a Novel Tyrosyl tRNA Synthetase Inhibitor from a *Micromonospora* sp.

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The compound designated SB-219383 is a potent and selective inhibitor of bacterial tyrosyl tRNA synthetases. It exhibits an IC50 of <1 nM against *Staphylococcus aureus* tyrosyl tRNA synthetase and weak *in vitro* activity against Staphylococci and Streptococci. Here we present data consistent with SB-219383 eliciting an amino acid starvation in both *S. aureus* and *Streptococcus pneumoniae*, supporting the conclusion that the antibacterial activity of SB-219383 is due to tyrosyl tRNA synthetase inhibition.

Aminoacyl tRNA synthetases are enzymes responsible for charging their cognate tRNA(s) with the correct amino acid. The enzymes are essential for cell viability and so represent a large group of potential targets for antibacterial agents. An example of such an agent is pseudomonic acid A which is a naturally occurring antibiotic that inhibits isoleucyl tRNA synthetase. While numerous other inhibitors of tRNA synthetases are known[1,2], only pseuodomonic acid A is used therapeutically.

During a screen of soil microorganisms for inhibitors of *Staphylococcus aureus* tyrosyl tRNA synthetase (YRS) a novel compound designated SB-219383 was detected[3,3a]. This compound is a potent, competitive inhibitor of YRS but exhibits only weak antibacterial activity. We wished to determine if the weak antibacterial activity observed was due to YRS inhibition. In this paper we show that treatment of *S. aureus* and *Streptococcus pneumoniae* with SB-219383 leads to the induction of the stringent response which occurs in response to amino acid limitation. Given this is the expected response for a tRNA synthetase inhibitor, we conclude that the whole-cell activity of SB-219383 is due to inhibition of YRS.

Materials and Methods

Bacteria

The bacterial strains used in this study were *S. aureus* RN4220, *S. pneumoniae* R6 (wild-type), and *S. pneumoniae* Δ*rel*[4]. *S. pneumoniae* strains were grown in Todd Hewitt broth +0.5% yeast extract (THY) supplemented with 1,000 units/ml catalase. Frozen cultures of each strain were prepared by inoculating 5.0 ml medium with a single colony from a Tryptic Soy Agar +5% blood agar plate. The cultures were grown statically at 37°C in 5% CO_2 to an A_{550} of 0.3. Glycerol was then added to 12% and the cultures were rapidly frozen on dry ice in 200 μl aliquots.

Nucleotide Analysis

The effect of SB-219383 on intracellular nucleotide pools of *S. aureus* was measured as described previously[5] except cultures were grown in LB broth.

Macromolecular Synthesis

Monitoring the incorporation of radiolabeled precursors into macromolecules (macromolecular synthesis profiling or MMS) was performed with *S. pneumoniae* strains. Similar experiments were carried out each with a different radiolabeled precursor. Tubes containing different concentrations of SB-219383 or pseudomonic acid A were prepared and set in a 37°C water-bath. To tubes containing no drug 3.0 μCi [^{14}C] isoleucine or 1.5 μCi [^{14}C] uridine was added. A 3.2 ml culture was then added to the tubes containing radiolabel. At 3 and 6 minutes, 80 ul was removed to 80 ul ice-cold 10% TCA in a microtiter plate.

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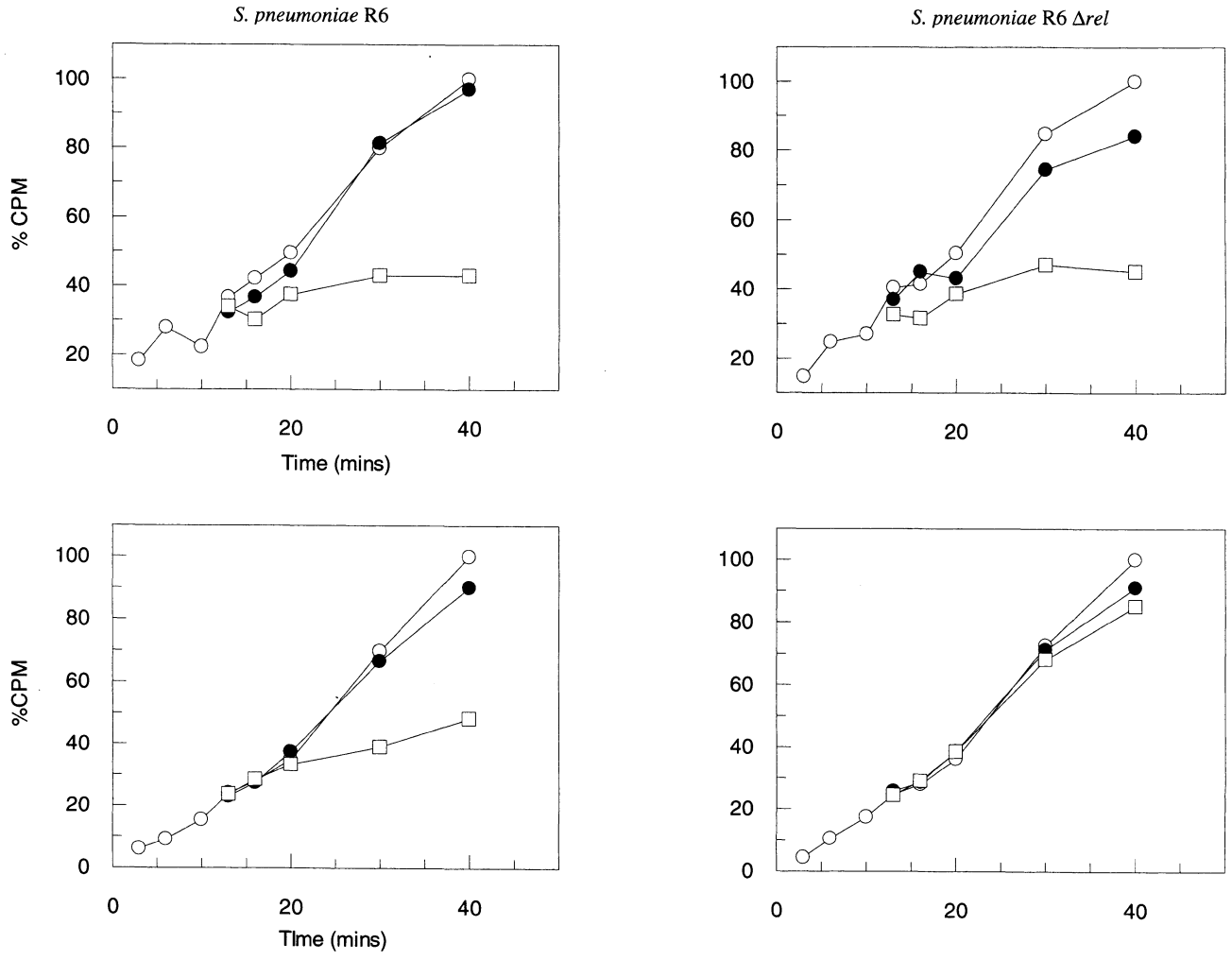
At 10 minutes another sample was taken and 1.0 ml culture was removed to each of the 2 drug-containing tubes. Samples from all tubes were taken at 13, 16, 20, 30 and 40 minutes. TCA precipitates were harvested onto a GF/C 96-well filter plate using a Packard Filtermate 196 Harvester 30 minutes after the last time point and the plate was allowed to dry overnight. After adding 50 μl of Microscint 20 to each well, radioactivity was measured using a Packard Topcount. The incorporation of precursors was plotted after normalization with the untreated control.

Results and Discussion

To determine if SB-219383 causes YRS inhibition *in vivo*, we measured its effect on the physiology of treated bacterial cultures. A YRS inhibitor should lead to an increase in uncharged tRNA, resulting in the inhibition of translation. Additionally, starvation for an amino acid elicits the stringent response, a complex change in the physiology of the bacterium[6]. The most notable feature of the stringent response is an abrupt decrease in the rate of stable RNA synthesis. This decrease can be easily measured as a rapid decrease in uridine incorporation. The stringent

Fig. 1. Incorporation of isoleucine and uridine following treatment with SB-219383 in *Streptococcus pneumoniae* strains.

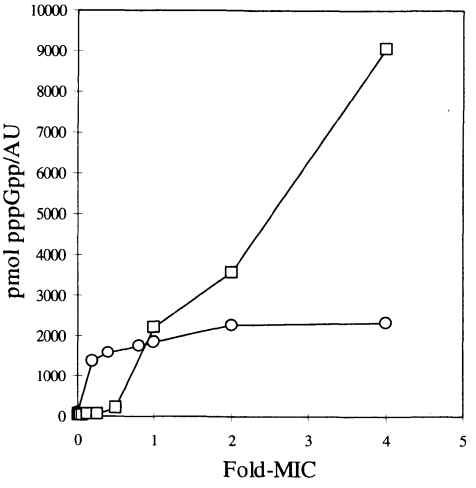


Cultures were labeled with either ^{14}C leucine or ^{14}C uridine as described in Materials and Methods. Incorporation into TCA precipitable material was monitored following treatment with compound in *S. pneumoniae* R6, left panels, and *S. pneumoniae* Δ*rel*, right panels. Top panels, incorporation of isoleucine. Bottom panels, incorporation into uridine. The concentrations of SB-219383 were no drug, open circles; 35 μg/ml, closed circles; and 70 μg/ml, open squares.

response is mediated by one or both of the nucleotides guanosine 3′,5′-bis(diphosphate) and guanosine 3′-diphosphate, 5′-triphosphate, known collectively as (p)ppGpp. Strains that lack the ability to synthesize (p)ppGpp do not exhibit an abrupt decrease in stable RNA synthesis in response to amino acid starvation. In *S. pneumoniae* and *S. aureus*, (p)ppGpp synthesis is catalyzed by the product of the *rel* gene[4,7]. We measured the effect of SB-219383 on uridine incorporation in *S. pneumoniae* and an isogenic *S. pneumoniae* Δ*rel* strain. As shown in Fig. 1, treatment of both strains with SB-219383 resulted in inhibition of isoleucine incorporation, indicative of inhibition of translation. Uridine incorporation, however, was only inhibited in the wild-type strain and not in the Δ*rel* strain. This pattern was also seen when the same strains were treated with pseudomonic acid A[4]. From these results we conclude that SB-219383 elicits a stringent response in *S. pneumoniae*.

In order to verify that SB-219383 results in an amino acid starvation in *S. aureus* we measured its effect on intracellular pppGpp pools. For *S. aureus* this approach gives clearer results than measuring uridine incorporation because a Δ*rel* derivative of *S. aureus*, which is necessary for an unambiguous interpretation of the uridine incorporation results, is not available. It has recently been shown that *rel* is an essential gene in for *S. aureus*[7]. Cultures of *S. aureus* were treated with SB-219383 and then their nucleotides were extracted with alkali. The resulting extracts were analyzed by HPLC. Pseudomonic acid A was used as a control. As previously reported[8], *S. aureus* was found to accumulate only pppGpp. As shown in Fig. 2, both SB-219383 and pseudomonic acid A elicited an increase in pppGpp levels. This leads us to the conclusion that SB-219383 elicits a stringent response in *S. aureus* as it does in *S. pneumoniae*. Given the potent inhibitory activity of SB-219383 against YRS, the induction of the stringent response by SB-219383 is most likely caused by decreased acylation of tyrosyl-tRNA. Interestingly, pseudomonic acid induced increases in pppGpp pools at sub MIC levels, while the increase in pppGpp levels in response to SB-219383 occurred at MIC or higher. This result may demonstrate differing penetration properties of the two compounds and also explain the discrepancy between the target potency and antibacterial activity of SB-219383.

Fig. 2. Accumulation of pppGpp in *Staphylococcus aureus* following treatment with SB-219383.



Cultures of *S. aureus* were treated with SB-219383 or pseudomonic acid A and extracted as described in Materials and Methods. Concentrations of pppGpp were determined following HPLC separation of nucleotides and are plotted versus MIC concentrations. Open circles, pseudomonic acid A treated cultures; open squares, SB-219383 treated cultures.

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