The effect of antibiotic treatment on the intracellular nucleotide pools of *Staphylococcus aureus*

Rebecca C. Greenwood, Daniel R. Gentry *

GlaxoSmithKline, 1250 S. Collegeville Road, Collegeville, PA, 19426-0989 USA

Received 12 November 2001; received in revised form 5 December 2001; accepted 20 December 2001

First published online 1 February 2002

Abstract

In an assessment of antibiotic action on *Staphylococcus aureus*, we found that distinct changes in intracellular nucleotide pools occur depending on the antibiotic mode of action. In particular, we have quantitated the effect of antibiotics on pools of the nucleotide guanosine 3'-diphosphate, 5'-triphosphate (pppGpp). Intracellular pppGpp levels increased in response to treatment with the isoleucyl tRNA synthetase inhibitor mupirocin, the uncoupler carbonyl cyanide-m-chlorophenylhydrazone, and rifampicin. These compounds were distinguishable by the degree in which they increased the pppGpp pool and by their differential effect on the pools of other nucleotides. This technique has been used to confirm and to refute the expected mode of action of several compounds identified as possible inhibitors of tRNA synthetases. Our results provide the framework for using nucleotide analysis in the assessment of novel antimicrobial compounds with unknown modes of action. ß 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: High-performance liquid chromatography; Nucleotide; pppGpp; Antibiotic; *Staphylococcus aureus*

1. Introduction

Current efforts to identify compounds with antibiotic activity include both target-based approaches and empirical screening of compounds for whole cell activity. While both approaches have their challenges, it is particularly difficult to identify the target of a compound with whole cell activity with no knowledge of that compound’s mode of action. This is important because once the target inhibited by a bacterial compound is identified a biochemical assay can be developed which in turn would allow for improvement in the compound through structure-activity relationships. Typical means of finding the mode of action of compounds include both genetic and physiological approaches. Genetic approaches can be tedious and still not identify the true target, while physiological approaches, such as measurement of the differential change in incorporation of macromolecular precursors, are often incomplete and ambiguous. We are therefore interested in additional assays to help elucidate the mode of action of compounds. One potentially useful technique is the analysis of nucleotide pools following treatment with compound. It is well known that compounds that induce nutrient starvation conditions lead to the accumulation of the nucleotides guanosine tetraphosphate and/or guanosine pentaphosphate [1]. Also, compounds that interrupt respiration are normally expected to have an impact on adenylate nucleotides. Finally, by cataloging the effects of compounds with known antibiotic action on nucleotide pools the effect of compounds with unknown mode of action may be suggested. In this paper we describe our efforts in assessing the effect of known antibiotics on nucleotide pools in *Staphylococcus aureus*. In particular, we have quantitated by high-performance liquid chromatography (HPLC) the effect of compounds on pppGpp pools. This work provides a framework for the future development of nucleotide analysis for the assessment of antibiotic mode of action.

2. Materials and methods

2.1. Strains and growth conditions

The commonly used laboratory strain *S. aureus* RN4220 was used throughout this study. Results are essentially the...
same for limited experiments using a clinical isolate of *S. aureus* (data not shown). Strain RN4220 was grown in THY (Todd–Hewitt broth with 0.2% yeast extract, both from Difco) at 37°C. The minimum inhibitory concentration (MIC) of various antibiotics against this organism are (µg ml⁻¹): mupirocin, 0.125; chloramphenicol, 8.0; cerulenin, 128; carbonyl cyanide-m-chlorophenylhydrazone (CCCP), 1.0; ciprofloxacin, 0.5; kanamycin, 4; rifampicin, 0.0156; erythromycin, 0.5.

2.2. Preparation of extracts

Extracts were prepared as described by Little and Bremer [2] with minor modifications. An overnight culture of RN4220 grown at 37°C in THY was diluted 1/1000 in THY. The culture was grown to an OD₆₀₀ nm of 1.0. Aliquots were treated with 5× MIC compound and incubated at 37°C for a further 30 min. The cells were fixed by adding 1.0 ml 1.9% formaldehyde and incubated on ice for 30 min. The fixed cells were then pelleted and washed once with 10 ml phosphate-buffered saline (PBS). The PBS-washed pellet was then re-suspended in 1.0 M KOH and incubated on ice for 30 min, after which 88% H₃PO₄ was added to neutralize the extract (usually 2–3 µl). Prior to HPLC analysis, the extracts were subjected to microcentrifugation at 4°C for 15 min and then passed through 4.0-mm 0.45 µM filter.

2.3. Assay of nucleotide pools

HPLC analysis of nucleotides was performed with a Beckman System Gold HPLC system using a Regis Little Champ C₁₈ column. Buffer A consisted of 30 mM KH₂PO₄, 10 mM PicA reagent (Waters), pH 6.0. Buffer B was acetonitrile. Nucleotides were separated at 1.0 ml min⁻¹ using a concave gradient (curve value of 4) of 100% buffer A to 40% buffer B over 60 min, followed by 5 min at 40% buffer A. Nucleotides were detected using a photodiode array detector at 254 nm.

2.4. Chemicals

Nucleotide standards for commercially available compounds were purchased from Pharmacia (nucleotide triphosphates) or Sigma (nucleotide diphosphates). ppGpp and pppGpp were synthesized from ATP and GTP or GDP using ATP nucleotide pyrophosphate kinase partially purified from *Streptoverticillium morookaensis* [3]. Following extraction with water-saturated phenol, the nucleotides were purified by anion-exchange chromatography on QAE Sepharose essentially as described by Cashel [4]. Intracellular concentrations of pppGpp were measured by comparison to a standard curve using pure pppGpp. The level of detection using this method is about 5 pmol.

Fig. 1. HPLC separation of highly phosphorylated nucleotides from *S. aureus* following treatment with various antibiotics. Cultures of *S. aureus* were grown, treated, and extracted as indicated in Section 2. Shown are HPLC chromatograms using OD₂₅₄ as detection. A: Treatment with 5× MIC mupirocin. B: Treatment with 5× MIC CCCP. C: Treatment with 5× MIC rifampicin. Solid line, untreated control culture; broken line, treated culture. MIC values are reported in Section 2.
3. Results and discussion

The technique we chose for extraction of nucleotides is very similar to that of Little and Bremer [2]. As observed by Little and Bremer [2], we found that lysis with KOH was much more efficient than extraction with formic acid [6] and that fixing with formaldehyde was very effective in reducing apparent degradation of nucleotides subsequent to extraction. Also, fixing the cells with formaldehyde allowed for the cell pellets to be washed. Without washing, nucleotide analysis of extracts of cultures grown in complex media proved to be difficult due to interfering substances found in the culture medium. For the limited number of drugs tested, we obtained similar results using 32P-labeling of cultures followed by thin-layer chromatography of formic acid extracts. Using both techniques, we observed only the accumulation of pppGpp following mupirocin and other tRNA synthetase inhibitors. This is in agreement with Cassels et al. [5] but at odds with Crosse et al. [6], who observed the accumulation of ppGpp and ppGp but no pppGpp.

The results of the effect of various antibiotics on nucleotide levels are shown in Figs. 1A–C and 2. As expected and reported previously [5], the isoleucyl tRNA synthetase inhibitor mupirocin induces the accumulation of pppGpp with no ppGpp detected. A concurrent increase in CTP also occurs after treatment with mupirocin. Though obscured by a decrease in GTP pools, which occurs transiently following induction of the stringent response [1], UTP pools also appear to increase in response to mupirocin. This is probably due to the inhibition of stable RNA transcription by pppGpp leading to the accumulation of these two transcription substrates. The RNA polymerase inhibitor rifampicin leads to a clear increase in RNA precursors (Fig. 1C), distinguishing it and likely other RNA polymerase inhibitors from compounds that will illicit a stringent response [1]. UTP pools also appear to increase in response to mupirocin. This is probably due to the inhibition of stable RNA transcription by pppGpp leading to the accumulation of these two transcription substrates. The RNA polymerase inhibitor rifampicin leads to a clear increase in RNA precursors (Fig. 1C), distinguishing it and likely other RNA polymerase inhibitors from compounds that will illicit a stringent response [1].

Nucleotide analysis showed that treatment with the compound SB-219383 identified as a potent inhibitor of tyrosyl tRNA synthetase [11]. Nucleotide analysis was used, in part, to confirm that the antibacterial activity SB-219383 is due to tRNA synthetase inhibition [12]. Conversely, a second compound was identified as an alanyl tRNA synthetase inhibitor. Nucleotide analysis showed that treatment with this compound yielded a pattern unlike a tRNA synthetase inhibitor but one very similar to CCCP, leading us to conclude that the antibacterial activity of this compound was due to an uncoupling effect. Measurement of the effect of the compound on S. aureus membrane potential using a protocol modified from that reported by Novo et al. [13] supported this conclusion.

Fig. 2. Quantitation of intracellular pppGpp pools in S. aureus following treatment with various antibiotics. Levels are presented as pmol per OD254 unit of extract. Levels were determined from extracts run in triplicate.

tRNA synthetases has yielded a number of compounds with antibacterial activity. The technique described here has been used as one means of confirming that the antibacterial mode of action of tRNA synthetase inhibitors coming from this screen is due to tRNA synthetase inhibition. For example, the compound SB-219383 was identified as a potent inhibitor of tyrosyl tRNA synthetase [11]. Nucleotide analysis was used, in part, to confirm that the antibacterial activity SB-219383 is due to tRNA synthetase inhibition [12]. Conversely, a second compound was identified as an alanyl tRNA synthetase inhibitor. Nucleotide analysis showed that treatment with this compound yielded a pattern unlike a tRNA synthetase inhibitor but one very similar to CCCP, leading us to conclude that the antibacterial activity of this compound was due to an uncoupling effect. Measurement of the effect of the compound on S. aureus membrane potential using a protocol modified from that reported by Novo et al. [13] supported this conclusion.

References


