Novel synthetic molecules targeting the bacterial RNA polymerase assembly

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Objectives: Despite extensive functional screening of the bacterial RNA polymerase (RNAP) over the past years, very few novel inhibitors have been reported. We have, therefore, decided to screen with a radically different, non-enzymic, protein–protein interaction assay. Our target is the highly conserved RNAP–s interaction that is essential for transcription.

Methods: Small molecule inhibitors of the RNAP–s interaction were tested for their activity on transcription and on bacteria.

Results: These compounds have antibacterial activity against Gram-positive bacteria including multi-resistant clinical isolates.

Conclusions: This is, to our knowledge, the first example of a small molecule inhibitor of this interaction.

Keywords: transcription, antibacterial drug screening, antibacterials, antibiotic resistance, anti-infective agents

Introduction

The advent of bacterial genomics has provided data and bioinformatic tools to rationally identify potentially novel antibacterial targets. However, target validation remains a limiting step in the antibacterial agent discovery process.

The RNA polymerase (RNAP) is a key enzyme that is essential for bacteria growth and has already been targeted by the commercial antibiotic rifampicin. In bacteria, RNAP is a multi-subunit complex composed of α2ββ′ (the core enzyme), interacting with σ to form the holoenzyme. Rifampicin binds in a pocket of the RNAP β-subunit deep within the DNA/RNA channel 12 Å from the catalytic site and probably interferes with RNA extrusion. Other antibiotics not in therapeutic use also target the RNAP. Among these molecules, σ factors are a family of proteins involved in transcription initiation, whose functions are well defined. The σ factors bear the promoter selectivity of the polymerase. Following promoter binding, the σ–RNAP complex progresses through several intermediate complexes to a stable initiated open complex and then to an elongation complex. Among the σ factors, some like the Escherichia coli protein σ70, which promotes transcription from genes expressed in exponentially growing cells, are essential for the viability of the bacteria.

The complete genome sequences of pathogens such as E. coli, Haemophilus influenzae, Staphylococcus aureus and Enterococcus faecalis have now been obtained (http://www.tigr.org). Experimental results and sequence alignments of σ and RNAP...
sequences indicate that the interface between σ factors of the σ^{70} family and the core polymerase is extensively conserved among bacteria. Functionality is also conserved: in vitro transcription is often possible when a heterologous σ factor of bacteria is mixed with E. coli core.^{10-13} Such a high degree of conservation in bacteria and the absence of σ factors in mammalian cells reinforce the interest of identifying specific inhibitors of core–σ interaction. Small molecule inhibitors could serve as useful tools for determining the interest of this potential new site and may constitute a first step in the discovery of new valuable antimicrobial therapeutics.

We recently identified in a chemical library, synthetic compounds inhibiting σ–core interaction.^{14} The results presented here demonstrate that these compounds have IC_{50} values in the low micromolar range. The molecules inhibit the growth of a wide range of Gram-positive bacteria such as S. aureus, Staphylococcus epidermidis, Bacillus cereus and Bacillus anthracis, Streptococcus pneumoniae and some Gram-negative bacteria.

Materials and methods

ELISA dissociation assay

The screening assay used to identify small molecule inhibitors of σ–core interaction is based on the inhibition of core binding to σ^{70} by various compounds in solution.^{15} Briefly, 96-well microtitre plates were coated overnight at 4°C with σ^{70} (300 nM) in PBS. After washing with PBS/0.1% (v/v) Tween 20 (PBS/Tween 20), the plates were saturated with 200 μL of PBS/Tween 20/1% (w/v) BSA, for 1 h at room temperature. The compounds pre-dissolved in dimethyl sulphoxide were diluted to 10 μM in 0.1 mL of PBS/Tween 20 containing 10 nM of core polymerase. The mixture was transferred to the microtitre plates and incubated at room temperature for 30 min. The plates were extensively washed, and the binding of core to σ was quantified by addition of the 11D11 anti-core antibody–peroxidase conjugate^{16} and the substrate–chromogen mixture (o-phenylenediamine and H_2O_2). For evaluation of the IC_{50}, core was incubated under the same experimental conditions with serial dilutions of the compounds. The values were calculated using the LSW data analysis toolbox (MDL, San Leandro, CA, USA).

MIC measurements and cytotoxicity assay

 MICs were determined according to the CLSI guidelines.^{16,17} Bacterial clinical isolates were classified on the basis of their susceptibility to antibiotics according to the guidelines of the CLSI.^17 The strains used in this study were Gram-positive bacteria [S. aureus (CIP 76.25) S. epidermidis (CIP 68.21), S. pneumoniae (CIP 103566), B. cereus (ATCC 14579), B. anthracis (non-pathogenic strain 9131)] and Gram-negative bacteria [E. coli (CIP 76.24), Pseudomonas aeruginosa (CIP 76.110), Bacteroides sp. (clinical isolate from the CHU of Nîmes, France), Pasteurella multocida (DSM 5881)]. The MIC values were determined in duplicate by a broth microdilution method in 96-well microtitre plates. The inoculum was prepared from colonies grown overnight in Mueller–Hinton broth and diluted to 10^8 cfu/mL. The concentrations of the antimicrobial agents tested ranged from 33 to 0.062 mg/L. Anaerobic bacteria were incubated in a Genbag anaer (BioMérieux, Marcy l’Étoile, France). The microtitre plates were incubated at 37°C and were examined for growth after 24 h. MIC was defined as the lowest concentration that inhibited visible growth.

Susceptibility of Mycobacterium tuberculosis (clinical isolate from the CHU of Nîmes, France) was tested in Mycobacteria Growth Indicator Tubes (MGIT; BBL Becton Dickinson Microbiology systems, Le Pont de Clai, France). The inoculum was prepared from a M. tuberculosis clinical isolate by subculture on Löwenstein–Jensen slants. The colonies were transferred to 5 mL of sterile water and vortexed for 20 min at 37°C; then the suspension was diluted 10^{-2} and 10^{-4}. Next, 0.1 mL of each dilution was added to 5 mL MGIT in the presence of the test compound at a concentration ranging from 30 to 0.062 mg/L. The cultures were examined every 3 days.

Filamentous fungi were grown at 30°C for 18 h in RPMI medium. Chinese Hamster ovary (CHO) cells were grown in RPMI medium supplemented with 5% fetal calf serum. The cells were incubated for 24 h at 37°C in the absence of serum and in the presence of the compounds, and the cytotoxicity was measured using the cell cytotoxicity kit I (Roche Applied Sciences, Meylan, France).

Bactericidal effects of SB2 on growing S. epidermidis

The S. epidermidis (CIP 68.21) inoculum was prepared from colonies grown overnight in Mueller–Hinton broth and diluted to 10^7 cfu/mL in Mueller–Hinton broth. As the MIC of rifampicin increases greatly at high inoculum density, rifampicin was used at six times its MIC measured under standard conditions (MIC = 0.007 mg/L); SB2 was used at twice its MIC. The bacteria were treated for 0–40 min with the antibiotic, and then diluted 1 : 100. The density of viable bacteria was measured by plating at several dilutions. The measurements are an average of three independent experiments.

Transcription assay

E. coli core RNAP or holoenzyme (100 nM) (Epiconet, Madison, USA) activities were assayed in the following mixture (final volume of 50 μL): 25 mM Tris, pH 7.9, 10 mM MgCl₂, 1 mM EDTA, 0.2 mM CTP, 0.2 mM GTP, 0.2 mM ATP, 0.05 mM UTP, 1 μCi [3H]UTP, pGEM-β-gal templates, 1 mM DTT, 0.1% (w/v) of BSA and 250 mM NaCl. The inhibitor was added, and the samples were incubated at 37°C for 10 min and quenched with 2 mL of cold 5% trichloroacetic acid on ice. Each sample was filtered onto Whatman GF/C filters, washed with 5% trichloroacetic acid and with ethanol, dried and counted by liquid scintillation. The T7 polymerase transcription assay was performed using the same conditions with 25 units of T7 RNAP and 200 ng of pET21a plasmid.

Labelling of macromolecules in S. epidermidis

Bacteria were cultured in supplemented MOPS liquid medium in triplicate. Radioactive precursors (1 μCi/ml) were added during the early logarithmic phase and 1 min before the addition of inhibitors at two times their MICs. Incorporation of radioactivity into macromolecules was determined by following previously published procedures 18,19 Briefly, bacteria (0.1 mL) were treated with 2 mL of trichloroacetic acid (5%, w/v) followed by collection of filtrates on glassfibre filters (Whatman GF/C) and quantification of radioactivity by liquid scintillation counting. For each set of experiments a specific inhibitor of the pathway of interest was included as a positive control.

Results

Inhibitor discovery

Multiple domains and complex allosteric regulations are involved in the binding of core to σ.^{20,21} Our assay uses σ^{70} and core instead of truncated proteins, and allows the direct detection of σ–core interaction, independently of the polymerase activity of the complex.^{14,22}
Using this assay we identified several compounds [ref. (14) and Table 1] that inhibited σ–core interaction. The compounds characterized here are inhibitors in a dose-dependent manner (Table 1) in several independent experiments. Their IC50 values in our in vitro binding assay were in the low micromolar range: 2 μM for SB2 and SB8 in the ELISA. As shown in Figure 1, even at a concentration one order of magnitude higher than the IC50, SB2 specifically inhibited the binding of σ70 to core but did not affect the interaction of core with the antibody or the interactions between the different subunits of core. The specificity of the molecule was also verified in different assays with irrelevant proteins: the absence of interference of core recognition by four different monoclonal antibodies whose affinities are in the same range as the σ–core interaction (Kd = 10–9 M); and the absence of inhibition of E. coli DNA ligase activity, human thyroid peroxidase activity and in vitro transcription–translation in reticulocyte lysate were unaffected by 50–100 μM concentrations of SB2 (data not shown).

Table 1. Inhibition of the interaction between core RNAP and σ70

<table>
<thead>
<tr>
<th></th>
<th>SB2</th>
<th>SB4</th>
<th>SB5</th>
<th>SB7</th>
<th>SB8</th>
<th>SB12</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC50 (μM)</td>
<td>2 ± 2</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>3 ± 2</td>
<td>1.8 ± 3</td>
<td>&gt;100</td>
</tr>
<tr>
<td>MIC for S. aureus (CIP 76.25) (mg/L)</td>
<td>2</td>
<td>&gt;33</td>
<td>&gt;33</td>
<td>16</td>
<td>2</td>
<td>&gt;33</td>
</tr>
</tbody>
</table>

The IC50s are shown as means ± SD of three independent experiments and were obtained as described in the Materials and methods section.

Figure 1. Immunoprecipitation of the core σ70 in the presence of SB2. PBS/Tween 20 (0.1 mL) without core (A), with 5 pmol of core (lanes B–E) and with 15 pmol of σ70 (lanes C–E), was incubated for 30 min without SB2 (lane C) or with 10 μM (lane D) or 20 μM (lane E) SB2.

Figure 2. SB2 is a transcription inhibitor. The transcription assay was performed using core polymerase and poly(dA–dT) (open squares) or pGEM-β-gal (filled squares) templates at the concentrations of SB2 indicated in the Materials and methods section. For T7 transcription a pET21a plasmid was used as template (filled circles). Mean values ± SD of triplicate determinations are shown.
Antibacterial activity of SB2

We observed a close relationship between the activity of the molecules in the ELISA assay and on bacteria. Structure–activity data presented in Table 1 resume the analysis made from the iterative selection of more than 300 analogues. They demonstrate that SB2, SB7 and SB8 inhibited σ-core interaction and growth of S. aureus. These molecules are relatively hydrophobic for an antimicrobial (clogP = 2.9) and the presence of serum strongly impaired their activity (data not shown). Other compounds closely related to SB2, but without a negative charge on the aromatic ring (SB4, SB5 and SB12), did not disturb the interaction and had no effect on the growth of S. aureus. Compounds with less hydrophobic constituents on the nitrogen atom of the thioxothiazolidinone heterocycle (i.e. SB7) inhibited the interaction between core and σ in the same range of concentrations as SB2, but were inactive against these bacteria.

The effects of SB2 on macromolecular synthesis have been tested to evaluate its specificity on growing bacteria (Figure 3). In vivo, at low concentrations of SB2 (2-fold the MIC), a rapid inhibition of RNA synthesis occurred whereas DNA synthesis was not affected during a period of 2 h (about one generation, when the bacteria are growing at 25°C). Protein synthesis was affected only after 2 h. At higher concentrations (20-fold the MIC) the specificity was apparently lost; DNA, RNA and protein synthesis were affected earlier without any selectivity.

The in vitro activity of SB2 and its analogues on transcription was compared with their ability to inhibit the growth of several Gram-positive and Gram-negative bacteria (Table 2). In parallel, their toxicity was tested on eukaryotic cells. SB2, SB7 and SB8 had no effect on the growth of CHO cells. SB2 and SB8 had no effect on the growth of Candida albicans or Aspergillus fumigatus (SB7 was not tested). SB2, SB7 and SB8 exerted an antibacterial effect against S. pneumoniae, S. epidermidis, S. aureus, B. anthracis and B. cereus between 0.1 and 16 mg/L. For Gram-negative species, an antibacterial activity was observed against P. multocida.

Table 2. MICs of novel RNA polymerase inhibitors against bacteria and fungi

<table>
<thead>
<tr>
<th></th>
<th>SB2 (mg/L)</th>
<th>SB8 (mg/L)</th>
<th>SB7 (mg/L)</th>
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<tbody>
<tr>
<td><strong>Gram-negative bacteria</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>&gt;33</td>
<td>&gt;33</td>
<td>&gt;33</td>
</tr>
<tr>
<td>E. coli D22</td>
<td>2</td>
<td>2</td>
<td>&gt;33</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>&gt;33</td>
<td>&gt;33</td>
<td>&gt;33</td>
</tr>
<tr>
<td>P. multocida</td>
<td>4</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Bacteroides sp.</td>
<td>1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Gram-positive bacteria</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Streptococcus pneumoniae</td>
<td>2</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>S. epidermidis</td>
<td>1</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>S. aureus</td>
<td>2</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>B. anthracis</td>
<td>0.1</td>
<td>0.1</td>
<td>4</td>
</tr>
<tr>
<td>B. cereus</td>
<td>0.3</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>M. tuberculosis</td>
<td>&gt;33</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Eukaryotic cells</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. albicans</td>
<td>&gt;33</td>
<td>&gt;33</td>
<td>ND</td>
</tr>
<tr>
<td>A. fumigatus</td>
<td>&gt;33</td>
<td>&gt;33</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, not determined.

Figure 3. Labelling of macromolecules in S. epidermidis. (a) [3H]Uridine; (b) [3H]thymidine; (c) [3H]leucine. For each set of experiments, curves representing bacteria incubated without antibiotics (filled diamonds), with SB2 (filled triangles) and with a specific inhibitor (filled squares) of the pathway of interest are displayed [(a) rifampicin; (b) novobiocin; (c) minocycline]. Mean values ± SD of triplicate determinations are shown.
and against the anaerobic *Bacteroides* sp., however, no activity was observed against *E. coli* or *P. aeruginosa*. As the proteins used for the screening and the characterization of the molecules were from *E. coli*, the absence of an antibacterial effect against this bacterium could suggest that SB2 could not reach its intracellular target owing to the low permeability of the membrane. When the activity of SB2 was tested against *E. coli* D22 cells, a strain with a mutation in the *lpxC* gene, with increased outer membrane permeability, we observed antibacterial activity at 2 mg/L. This confirms that the molecules inhibiting the transcription activity of the *E. coli* RNAP in vitro can efficiently inhibit bacterial growth of permeable *E. coli* cells.

SB2 was also tested against a series of *Staphylococcus* clinical isolates resistant or multiresistant to antibiotics (Table 3). The MIC of SB2 for a reference strain and for these isolates was comparable despite their resistance to a wide range of commercial antibiotics and is worth mentioning that mutants resistant to rifampicin were found to be susceptible to SB2 (i.e. Sa13).

We decided to determine whether the mode of action of SB2 was bacteriostatic or bactericidal. In time-to-kill experiments performed at a concentration equal to twice the MIC (Figure 4) on growing *S. epidermidis*, SB2 was strongly bactericidal. A three order of magnitude decrease in the bacterial titre was observed after only 10–15 min of incubation; this was comparable to rifampicin. This demonstrates that the target of SB2 is essential for bacterial survival. We attempted to select spontaneous resistant mutants to SB2 at a concentration of 3× MIC without success, suggesting a mutation frequency <10^{-9}.

### Discussion

RNAP is a pivotal enzyme of the bacterial machinery, and σ^{70} is an essential transcription factor that confers sequence-specific recognition of the exponential phase promoters by the RNAP. However, despite extensive functional screening of the enzyme over the past years, very few novel inhibitors have been reported since the discovery of rifampicin. Most of these molecules are natural products sharing a similar binding site and frequently show cross-resistance. Only recently a new compound, which, according to the authors, inhibits nucleotide addition by hindering movements of active site structures was discovered using a classic transcription assay, suggesting that other sites on the enzyme could be targeted by a drug.
In order to increase our chance of targeting new regions on the core polymerase we decided to screen with a radically different protein–protein interaction assay. Employing a robust ELISA of E. coli core polymerase binding to immobilized ασ0, we discovered low molecular weight compounds as potent inhibitors of the E. coli RNAP. These drugs inhibited the binding of σ0 to core in a dose-dependent manner, with an IC50 of 2–15 μM. This inhibition of interaction was confirmed by an immunoprecipitation assay, which demonstrated that SB2 effectively inhibited the binding of σ0 to core. This inhibition was specific: neither the integrity of assembly between α, β and β’ nor the binding of five monoclonal antibodies of comparable affinities directed against core was affected by concentrations of SB2 10–50-fold greater than the IC50. This is, to our knowledge, the first example of a small molecule inhibitor of this interaction.

When tested for its ability to inhibit transcription, SB2 turned out to be an effective transcription inhibitor. Moreover, it not only inhibited σ-dependent transcription but also σ-independent transcription on poly(dA–dT), which proves that the target is on the polymerase side. The σ-dependent transcription of a lac promoter is more sensitive to the inhibitor than σ-independent transcription. This could be consistent with a non-competitive, allosteric inhibition. However, the complex nature of the transcription cycle precludes rigorous interpretation of these results as discussed elsewhere.

In drug discovery, mechanisms of action predicted from enzymic study do not necessarily match the mechanisms of action in vitro. By following macromolecular synthesis in growing S. epidermidis, we observed a pattern of inhibition very similar to that obtained for known inhibitors of RNAP. This inhibition rapidly and preferentially affects RNA synthesis, and is not due to an induction of the stringent response since the synthesis of proteins and DNA is not affected.

The antibacterial activity of the molecules was tested on Gram-positive and Gram-negative bacterial pathogens. SB2 showed good activity in vitro, in the micromolar range, against all the Gram-positive bacteria tested, including S. aureus, S. epidermidis, B. cereus and B. anthracis; the activity was lower against S. pneumoniae. Interestingly, SB2 and its analogues were also active against a wide range of isolates of Staphylococcus including rifampicin-resistant mutants. Our compounds were also active against several Gram-negative bacteria, such as Bacteroides sp., but were inactive against E. coli or P. aeruginosa. Using more permeable E. coli D22 bacteria that are partially deficient in lipopolysaccharide, we measured an antibacterial activity of 2 mg/L. This indicates that the difference in the inhibitory activity of SB2 against Gram-positive and the Gram-negative bacteria is not owing to variations in the affinity for the target but rather to the intrinsic permeability of the bacterial species.

Whether the effects in vitro are owing to direct competition or are allosteric, as recently shown for rifampicin, is under investigation, but this confirms that the site of action of SB2 is essential for polymerase activity. We cannot exclude that in vivo, the absence of spontaneous resistance may be owing to interaction of these relatively hydrophobic molecules with several targets; the absence of resistant mutants prevented us from providing definitive genetic proof that the preferential target of the molecules was the core, and it was recently observed that several arylalkylidene rhodanes interact with penicillin-binding proteins. However, the fast and selective transcription inhibition observed in vivo, and the similarities between the bactericidal kinetics of rifampicin and SB2 are comforting facts. Experiments are ongoing to solve the structure of the drug–target complex.

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Transparency declarations

No declarations were made by the authors of this paper.

References


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