Reversal of methicillin resistance in *Staphylococcus aureus* by thioridazine

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**Objectives:** Thioridazine has been shown to reverse oxacillin resistance in methicillin-resistant *Staphylococcus aureus* (MRSA) *in vitro*. The aim of this study was to investigate whether thioridazine alone or in combination with oxacillin affects the transcription of the methicillin resistance gene meca and the protein level of the encoded protein PBP2a.

**Methods:** Viability of MRSA was determined in liquid media in the presence of oxacillin or thioridazine alone or in combination. Transcription of meca was analysed by primer extension, and the protein level of PBP2a was analysed by western blotting in the presence of thioridazine and oxacillin.

**Results:** We observed an increased susceptibility of MRSA towards oxacillin in the presence of thioridazine compared with bacteria grown with oxacillin or thioridazine alone. Transcription of meca was reduced with increasing concentrations of thioridazine in the presence of a fixed amount of oxacillin. Furthermore, the protein level of PBP2a was reduced when bacteria were treated with the combination of oxacillin and thioridazine. The two drugs also affected the mRNA level of the β-lactamase gene, blaZ.

**Conclusions:** The present study indicates that reversal of methicillin resistance by thioridazine in MRSA may be explained by a reduced transcription of meca and blaZ, resulting in a reduced protein level of PBP2a.

Keywords: MRSA, phenothiazine, meca, PBP2a

**Introduction**

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a growing problem in countries all over the world.¹ Attempts to control resistance by hygienic measures and restriction of antibiotic use have not been very successful, and there is a shortage of new efficient drugs for the treatment of severe infections. Recent studies suggest that resistance towards traditional antibiotics can be reversed by non-antibiotics, i.e. drugs with antimicrobial activity that are registered for other purposes.²

Phenothiazines are used in the clinic as neuroleptic anti-psychotic drugs but have, for a long time, been known to have antimicrobial activities.³ Thioridazine, a phenothiazine derivate, has potential for therapeutic use in problematic infections caused by antibiotic-resistant bacteria. However, thioridazine has been shown to have cardiotoxic side effects and was withdrawn in 2005 (reviewed by Thanacoody⁴). Even so, thioridazine is interesting for therapeutic use as it was reported that the (S)-enantiomer ([S]-thioridazine) is less toxic⁵ and a better helper compound to antibiotics than the other thioridazine derivate (R)-thioridazine.⁶ In human macrophages, thioridazine inhibits intracellular growth of methicillin-susceptible *S. aureus*⁷ or kills intracellular MRSA at concentrations that are achievable in humans.⁸ The addition of thioridazine at concentrations below the MICs of conventional antibiotics results in the reduction of the MICs of the antibiotics⁹,10 and reversal of oxacillin (methicillin) resistance in MRSA in culture-based experiments *in vitro*.¹⁰ Oxacillin resistance in MRSA is based on the presence of the meca gene, which encodes the penicillin-binding protein, PBP2a.¹¹ Previous studies have shown that the gene is still present in reversed strains exposed to thioridazine,¹² but apart from this, the mechanism behind the reversal of oxacillin resistance in MRSA remains unexplained.
In *S. aureus*, resistance to β-lactam antibiotics is mediated by PBPs to which β-lactam binds poorly and a β-lactamase encoded by *blaZ* that hydrolyses penicillins. The transcription of *mecA* and *blaZ* is co-repressed by the regulators of the two regulators, *mecI* and *blaI*, which are almost identical and can replace each other. In the presence of antibiotics, the sensing is mediated by signal transduction via the two transmembrane inducers, MecR1 or BlaR1, which will result in proteolytic autocleavage of the cytoplasmic domains of these proteins. To perform this autocleavage, the transducers undergo acylation by the antibiotic that causes conformational changes in the molecule. The sensor–transducer molecules, MecR1 and BlaR1, cannot replace each other, and the induction of *mecA* expression via MecR1 is slower than via BlaR1. Autocleavage of the signal transducer is followed by cleavage of the cognate repressor, MecI or BlaI, respectively, and subsequent induction of the transcription of *mecA* or *blaZ*. In MRSA, MecI is mutated or not present, which results in a continuous transcription from the *mecA* promoter introducing the antibiotic resistance. Regulation of *mecA* is therefore solely under the control of the *blaI*/*blaR1* regulon.

The aim of this study is to investigate the molecular background for thioridazine-induced reversal of oxacillin resistance in MRSA. We hypothesize that the reversal effects of thioridazine on resistance in MRSA are related to changes in gene expression and activities of proteins involved in the resistance mechanism.

### Materials and methods

**Bacterial strains and growth media**

MRSA ATCC 33591 was routinely grown at 37°C with shaking in brain heart infusion (BHI) medium (Difco) and Mueller–Hinton medium and agar (BBL) for subculture and maintenance. The MRSA strain was resistant to oxacillin with an MIC of >256 mg/L and to thioridazine with an MIC of 16 mg/L. *Escherichia coli* strain TOP10 (Invitrogen) was grown in Luria–Bertani medium.

**Determination of SCCmec type**

SCCmec type was determined at Statens Serum Institut (Artillerivej 5, DK-2300 Copenhagen S, Denmark) as described by Kondo et al.

**mecI sequence analysis**

Chromosomal DNA was prepared from *S. aureus* by using a Fast Prep DNA Kit (BIO 101 systems; QBIogene) according to the manufacturer’s direction supplemented with a pre-treatment of 10 mg/L lysostaphin for 1 h at 37°C. The AureoList Web Server (http://genolist.pasteur.fr) was used for primer design. PCR amplification of the DNA sequences was performed with primers mecI 4 and mecI 5 (Table 1) to analyse the sequence of the meC gene. Tag DNA polymerase (Amplicon) was used for the amplification reaction, and the thermocycling conditions were those recommended by the manufacturer. DNA sequencing was performed by DNA Technology A/S (Risskov, Denmark) with primer mecI 4.

### Table 1. Primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′→3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mecI 4</td>
<td>TCACTGTCAATGAACTGAAAGT</td>
</tr>
<tr>
<td>mecI 5</td>
<td>GTGAATGTGAACCCGCTTTT</td>
</tr>
<tr>
<td>mecA 3</td>
<td>GGGGATTTGGATTAACTGTTGAGCAGG</td>
</tr>
<tr>
<td>mecA 4</td>
<td>GGGGATTTGGACGTTCAGTCATTTCTACTTCACC</td>
</tr>
<tr>
<td>mecA 5</td>
<td>ACCAAACCGCAACACTACAC</td>
</tr>
<tr>
<td>mecR1 1</td>
<td>CATATATGTGAACTGTTGAGA</td>
</tr>
<tr>
<td>blaZ 1</td>
<td>GGGGAATTCGGGCGCGTTGATTACATCAGT</td>
</tr>
<tr>
<td>blaZ 2</td>
<td>GGGGATCCCGCTTGGTAATCGAAGCAT</td>
</tr>
<tr>
<td>blaZ 3</td>
<td>CTGGCAGTGTGAACTGTTG</td>
</tr>
<tr>
<td>blaR1 1</td>
<td>TTAATATAGCTTGAATATAATG</td>
</tr>
</tbody>
</table>

*The underlined sequence is an EcoRI restriction enzyme site. The underlined sequence is a BamHI restriction enzyme site.*

**Growth and viability assays on MRSA**

For growth experiments, overnight cultures grown in Mueller–Hinton medium were diluted 1:100 in fresh BHI medium containing various concentrations and combinations of thioridazine (Sigma Aldrich) (0–128 mg/L) and oxacillin (hospital pharmacy) (0–64 mg/L) corresponding to 0–8 times and 0–1/4 times the MIC, respectively. For viability experiments, overnight cultures diluted in fresh BHI medium were grown to early exponential phase and exposed to thioridazine and oxacillin as described earlier. Growth and viability of the MRSA was followed by measuring the optical density at 600 nm (OD_{600}) of the culture or by determination of the numbers of cfu/mL by spreading 10-fold dilutions on BHI agar plates.

**Primer extension analysis**

MRSA cultures were grown to an OD_{600} of 0.2 and subjected to various concentrations and combinations of oxacillin and thioridazine. A control without drugs was included. Bacteria were collected after 20 min of treatment before growth differences were observed due to the antimicrobial effect of the drugs. Total RNA was prepared by using a hot acid-phenol procedure. RNA concentration was determined by measuring A_{260} on a NanoDrop (Saveen Werner). Primer extension analysis was performed as described previously by Podbielski et al. by using 10 μg of total RNA per reaction mixture. Primers mecA 5, mecR1 1, blaZ 3 and blaR1 1 (Table 1) labelled at the 5′ end with [γ-32P]ATP were used to detect mecA, mecR1-mecI, blaZ and blaR1-blaI transcription start sites. PCR fragments created by the primers mecA 3 + mecA 4 and blaZ 1 + blaZ 2 (Table 1) were cloned into plasmid pTVC-lac and transformed into the TOP10 E. coli strain. The purified plasmids were used as template for DNA sequencing reactions for mecA, mecR1-mecI, blaZ and blaR1-blaI.

**Preparation of protein extracts**

The extracellular proteins were extracted according to Frees et al. with a few changes. MRSA cultures were grown to an OD_{600} of 0.2 and subjected to various concentrations and combinations of oxacillin and thioridazine. A control without treatment was included. After 0.5 h of incubation with oxacillin and thioridazine, the cultures were centrifuged to separate bacteria from the media containing secreted proteins. The media from the different cultures

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were sterilized with a filter cartridge (0.22 μm) to remove any bacteria. The extracellular proteins were precipitated in an Erlenmeyer flask by adding one volume of ice-cold 96% ethanol and placed at 4°C overnight. Protein pellets were collected by centrifugation (10,000 g; 30 min; 4°C) and suspended in 50 mM Tris–HCl (pH 6.8).

Cellular protein extracts containing both cytosolic and membrane proteins were prepared from the bacteria pellet collected. Pellets were suspended in a lysis buffer containing SDS to release proteins associated with the membrane (0.1 M NaCl; 50 mM Tris–HCl, pH 7.5; 10 mM EDTA; 0.5% SDS). Bacteria were disrupted and cell debris removed by centrifugation (3500 rpm; 5 min).

Protein concentrations were determined by measuring A280 on a NanoDrop (Saveen Werner).

**Gel electrophoresis**

Samples to be analysed were mixed with 3× SDS loading buffer (180 mM Tris–HCl, pH 6.8; 6% SDS; 30% glycerol; 0.015% Bromophenol Blue; 15 mM EDTA; 0.3 M 1,4-dithiothreitol), boiled for 5 min and loaded on an 8% Tris/glycine–SDS–polyacrylamide gel (prepared from a 40:2 acrylamide/bis-acrylamide stock solution).

**Western blotting**

Immunoblots were performed by semi-dry blotting by standard procedures, using anti-PBP2a antibodies (generated from peptide YKIDGKWQKDKSW by Genscript). The secondary antibody was peroxidase-conjugated goat-anti-rabbit immunoglobulin G (DakoCytomation). Immunoblots were developed with Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer Life Sciences).

**Results**

**Classification of MRSA strain**

The MRSA ATCC 33591 strain was used in this study. The strain was SCCmec-typed, and the typing results classified the strain as SCCmec type III (contains ccrF and mec class A). This means that the strain contains the mecI gene, which represses the mecA transcription. Sequencing of the mecI gene of our strain revealed a C to T mutation at position 202 resulting in a stop codon (TAA) when compared with the sequenced N315 S. aureus strain (AureoList Web Server). This mutation is repeatedly found in mecI-positive MRSA strains. mecA transcription in the MRSA strain is therefore derepressed with continuous expression from the mecA and mecR1-mecI promoters.

**Increased susceptibility of MRSA to oxacillin**

To investigate whether thioridazine in combination with oxacillin increased the susceptibility of the MRSA strain to oxacillin, the strain was grown in liquid media with thioridazine or oxacillin alone or in combination. The addition of both thioridazine and oxacillin reduced the growth of the MRSA strain (data not shown) compared with none or only one of the compounds added. Thioridazine itself did not affect the growth of MRSA at the used concentrations.

Additionally, we performed viability assays using different concentrations of thioridazine alone or in combination with a fixed concentration of oxacillin. The results presented in Figure 1 showed that the growth of MRSA was affected by 16 mg/L oxacillin, but the bacteria were not killed when compared with MRSA assayed without thioridazine and oxacillin. The addition of increasing concentrations of thioridazine to the fixed concentration of oxacillin reduced the number of cfu, indicating that oxacillin in the presence of thioridazine killed MRSA at concentrations where thioridazine and oxacillin alone did not kill MRSA. After 13 h, the combination of 64 mg/L thioridazine and 16 mg/L oxacillin reduced the viability by 3 log10 units. Thioridazine alone at concentrations ≤64 mg/L did not affect the viability of the MRSA. At 128 mg/L thioridazine, MRSA was killed by the antimicrobial effect of thioridazine alone. After 13 h, the effect of thioridazine disappeared in a concentration-dependent manner and the viability increased. We speculate that this increase is caused by degradation of thioridazine, which is known to be sensitive to light and is metabolized. The results suggest that treatment with thioridazine in combination with oxacillin increases the susceptibility to oxacillin of MRSA and that re-administration of thioridazine is necessary.

**Transcription analysis of the mec regulon**

To investigate whether the increased susceptibility to oxacillin involved the methicillin resistance marker, mecA, we looked at the mRNA level of the mecA gene and the two regulators of the mecA gene, mecR1-mecI. RNA for the primer extension analysis was purified from bacteria exposed to drugs for 20 min. In this time frame, no effect on growth was observed for the chosen concentrations (data not shown). When adding increasing concentrations of thioridazine to the MRSA, the level of mecA mRNA was low and unaffected by the drug (Figure 2). The addition of oxacillin alone induced the expression of mecA by 13.5-fold. When adding thioridazine at increasing...
concentrations, the expression was reduced from 13.5- to 1.8-fold at 128 mg/L thioridazine. Similar results were observed for mecR1-mecI expression, where the mRNA level of mecR1-mecI was low and unaffected by thioridazine. In this case, we observed a 3-fold induction of the genes by oxacillin.

When the addition of thioridazine, the mRNA level of mecR1-mecI was reduced to a 0.93-fold induction at the highest concentration when compared with the control lane. These results indicate that combining thioridazine with oxacillin reduces the expression and thereby the mRNA level of mecA, mecR1 and mecI in the MRSA strain, when compared with oxacillin alone.

Expression of the PBP2a protein in MRSA

The protein level of PBP2a was analysed by western blotting. The analysis was performed on an extracellular fraction containing secreted proteins and a cellular fraction containing both intracellular and membrane-associated proteins. Protein for the western blotting analysis was extracted from bacteria exposed to drugs for 30 min. In this time frame, no effect on growth was observed for the chosen concentrations (data not shown). In the western blot, the PBP2a protein was induced after induction with oxacillin in both fractions (Figure 3). The protein level was reduced by the addition of thioridazine in both fractions with the largest difference observed in the extracellular fraction, where the protein level was reduced to 0.40-fold compared with 0.72-fold in the cellular fraction. The PBP2a protein is not present in the control or after addition of thioridazine alone corresponding to the low expression of the mecA gene in primer extension (Figure 2).

Regulation of the antibiotic resistance response

As the repressors of the mec and bla operons are known to regulate both promoter regions,18 we performed primer extension on the bla promoter regions in order to determine whether the genes were regulated in the same manner as the mec genes.

When adding thioridazine alone, the mRNA levels of the blaZ and blaR1-blaI genes were low and almost unaffected by the thioridazine (Figure 4). After the addition of oxacillin alone, the mRNA levels of the blaZ and blaR1-blaI genes were induced by 51- and 14-fold, respectively. In the presence of both thioridazine and oxacillin, the mRNA levels of the blaZ and blaR1-blaI genes were reduced with increasing concentrations of thioridazine. At the highest concentration of 128 mg/L, the RNA level of the blaZ and blaR1-blaI genes was reduced to a 1.53- and 0.68-fold induction, respectively, when compared with the control. The results indicate a similar response of blaZ, blaR1 and blaI to the addition of the combination of thioridazine and oxacillin, as in the case of the mec genes.

Discussion

In the present study, we have confirmed observations from previous experiments regarding the positive effect on the susceptibility of MRSA by thioridazine in combination with oxacillin. The viability experiments clearly show that the combination of oxacillin and thioridazine can reverse the antibiotic resistance in an MRSA.

In the MRSA strain used in this study, the repressor of mecA, MecI, is not functional due to a point mutation introducing a
stop codon in the mecI gene. We expect that the BlaI repressor interacts with the mec promoters in the MRSA (Figure 5a), resulting in the repression of transcription from the mec and bla promoters. In the presence of the β-lactam antibiotic, oxacillin, the membrane-bound sensor transducer, BlaR1, will undergo conformational changes and cleave BlaI, resulting in transcription from both promoter regions. The outcome of these changes is protection of the bacteria against the β-lactam antibiotics (Figure 5b). In our primer extension experiments, thioridazine together with oxacillin down-regulates the expression from both mec and bla promoters. We reason that BlaI performs this repression based on the known regulatory descriptions of the mec and bla regulons.

The addition of the combination of the neurotropic drug thioridazine and the β-lactam oxacillin leads to lower levels of specific RNAs and proteins as observed in our experiments. Thioridazine is known to intercalate into the membrane close to the polar/apolar interface in the lipid bilayer as well as intercalate in-between nucleic bases of DNA, resulting in inhibition of all DNA-based processes. Furthermore, thioridazine induces ultrastructural changes in MRSA such as affecting the structure of the cell envelope, resulting in bacterial lysis at clinically relevant concentrations.

We speculate that the addition of thioridazine affects processes occurring in the cellular membrane, such as protein conformational changes necessary for the cleavage reaction of the repressor, BlaR1, to occur resulting in the retention of the repressed scenario (Figure 5c). The action of thioridazine relieves the effect of oxacillin on the PBPs in the membrane, resulting in increased susceptibility of the MRSA. This is thought to be accomplished by reducing the level of PBP2a in the membrane and perhaps more notably by reducing the activity of the PBP2a protein. When the PBP2a level is lowered or the protein is inactive, the MRSA is not protected against oxacillin and the MRSA is killed.

Another phenothiazine, chlorpromazine, has a similar effect on the reversal of methicillin resistance in MRSA, however, not as prominently as thioridazine. Chlorpromazine is known to bind to cell membranes and partition into the lipid bilayer. De-pigmentation, bacteriostatic and bactericidal effects as well as an increase in the K+ contents of S. aureus were found in early studies by Kristiansen et al. High concentrations of chlorpromazine were observed to induce large mesosome-like structures and asymmetrical cell divisions. Both thioridazine and chlorpromazine affect processes occurring in the bacterial membrane, e.g. permeability, transport proteins and cell envelope. The observed effects of thioridazine and chlorpromazine on the membrane are not necessarily the same, but the outcome is in both cases the reversal of antibiotic resistance in the bacteria supporting our theory that the reversal is related to changes in the membrane permeability and viscosity, which in turn affects molecular processes in the membrane. Changes in membrane fluidity are known to be involved in alterations in physical processes of cell membranes such as carrier-mediated transport, enzyme activities, receptor binding, phagocytosis, endocytosis, cytotoxicity and cell growth. The significant reducing effect seen on the mRNA level of mecA may be a result of thioridazine partitioned in the membrane. Furthermore, our results show that the production of PBP2a is reduced, which correlates to the reduced gene transcription of mecA. As the protein level is not completely diminished, the activity of PBP2a must be reduced in order to explain the increased susceptibility to oxacillin mediated by thioridazine. It is known that penicillin modifies PBP2a by acylation of a serine residue, giving a difference in mass of 334 Da. We speculate that thioridazine may also affect the acylation process occurring in the membrane, resulting in an inactive form of the PBP2a protein. The possible reduced activity of PBP2a correlates with the alteration in the membrane caused by changes in membrane fluidity.

In summary, we speculate that thioridazine affects membrane stability, leading to conformational changes of the BlaR1 protein and a reduced level of PBP2a activity. The results indicate a potential future in the clinic for the combination of (S)-thioridazine and oxacillin to reverse resistance in problematic MRSA infections. These issues are being addressed in ongoing studies.

Figure 4. Primer extension showing the expression of blaZ and blaR1-blaI of MRSA exposed to different concentrations of thioridazine (0–128 mg/L) alone (lanes 1–7) or in combination with 16 mg/L oxacillin (lanes 8–14). The panel at the bottom shows that an equal amount of RNA was used for both primer extensions. The ratio is calculated as each lane (lanes 2–14) relative to the control lane (lane 1). Tz, thioridazine; OXA, oxacillin.
Funding

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Transparency declarations

None to declare.

References

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Figure 5. Model showing the possible consequences in (a) MRSA, (b) MRSA + oxacillin and (c) MRSA + oxacillin + thoridazine on mec and bla regulation. See text for further details. Tz, thoridazine; OXA, oxacillin.

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transmembrane BlaR signaling pathway in Staphylococcus aureus. 


