

THE ANTIBACTERIAL ACTIVITY AND MODE OF ACTION OF EXPERIMENTAL INHIBITOR

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ABSTRACT

The development of antimicrobial resistance in bacteria has led to serious health problems worldwide. Therefore, the need to search for new antimicrobials is essential as it has become an urgent necessity. In this study, two types of compounds, putative cell wall inhibitors (TT225) and DNA synthesis (nitroxoline derivatives) inhibitors that were discovered via structure based drug design and modification of existing scaffolds respectively were analysed for their antimicrobial activity and mode of action. Minimum inhibitory concentration (MICs) against *S.aureus* and *B.subtilis* was determined while the mode of action studies was confirmed by using *B.subtilis* antibiotic biosensors. Results reveal that TT225 and 2 nitroxoline derivatives (GIS213 and GISB4) showed inhibition activity against *S.aureus* whereas in case of *B. subtilis*, 13 nitroxoline compounds showed positive results. In addition, the *B.subtilis* biosensors has revealed that among the nitroxoline derivatives only GISB4 and GIS249_2 were successful to induce the DNA synthesis.

Keywords: Antibacterial activity; Putative cell wall inhibitors; Biosensor; Biomarkers

INTRODUCTION

There has been an alarming rise in the prevalence of multiple drug resistance (MDR) in bacteria which has been a particular problem in hospitals acquired pathogens such as *Staphylococcus aureus*, the coagulase-negative staphylococci and enterococcal species [1]. Antibiotic resistance in the community has also emerged and problems such as gastrointestinal infections caused by species of *Salmonella*, *Shigella* and *Campylobacter* continue unabated [2]. As such the spread of multidrug-resistant bacteria remains a great concern. New technologies such as genomic approaches, molecular biology, robotics and bioinformatics have radically transformed the process of discovering new drugs [3]. The advancement in technology in identifying the protein structure of potential antibacterial targets has also yielded opportunities for drug design.

Nitroxolines have been reported to possess antibacterial activity, possibly by inhibiting DNA synthesis. Nitroxoline belongs to the quinoline family of antibiotics which have previously been shown to possess antibacterial and uropathogens. [4, 5]. It is structurally similar to 8-hydroxyquinoline (8-HQ) whereby the 7-nitro group is replaced in the benzene ring of 8-HQ [4]. Nitroxoline has a broad spectrum of biological and biochemical activities. A set of analogues of nitroxoline compounds was made available by collaborators from Slovenia. The novel compounds, TT225 and nitroxoline derivatives were tested for their antibacterial activity against *S. aureus* SH1000 and *B. subtilis* 1S34 and their mode of action that they possess using *B. subtilis* biosensors. It is predicted that nitroxoline and its derivatives will affect DNA synthesis inhibition in a similar manner to 8-hydroxyquinoline to undergo DNA synthesis inhibition [6, 7] as 8-hydroxyquinoline has been shown to induce the *B. subtilis* DNA biosensor [8]. By doing this research, it may provide the opportunity to find a new antibacterial agent that will help in combating the infectious diseases that have become a worldwide problem.

EXPERIMENTAL

Bacterial strain and media

S. aureus (SH1000) was routinely cultured using Mueller Hinton (MH) broth while Luria bertani (LB) broth was used in *B. subtilis* 1S34. Both types culture were grown at 37°C overnight in aerobic conditions. The *B. subtilis* strains containing analysis. Each biosensor was grown overnight in LB broth at 37° C in aerobic conditions.

Antimicrobial susceptibility testing

MIC determinations were carried out according to the British Society for Antimicrobial Chemotherapy (BSAC) broth microdilution method (Andrews, 2001) which involved exposure of 10^4 colony forming units (cfus)/ ml to doubling dilutions of antibiotic in a 96 well plate which was subsequently incubated at 37°C in aerobic condition for 18 hours. The absence of turbidity in a well represented the inhibition of bacterial growth and the lowest concentration of drug that inhibited growth represented the MIC.

Identification of the mode of action of novel agents: B. subtilis antibiotic biosensors.

The *B. subtilis* biosensor strains were grown in Luria Bertani (LB) broth containing erythromycin at 5µg/ml to an OD_{600nm} of 0.2 at 37°C overnight. The 90 µl sample culture was incubated with shaking at 37°C. The duration of incubation varied between each biosensor strain. For *yorB*, *yheI* and *fabHB* (DNA, protein, fatty acid), the incubation duration was 3 hours, for *yvgS* (RNA promoter) this was 1.5 hours and *ypuA* (cell envelope promoter) it was 1 hour. 60 µl of 0.1 M citrate buffer (pH5) containing 8mM D-luciferin was then added to each of well. (The citrate buffer was a mixture of 4.7 volumes of 0.1 M citric acid with 15.4 volumes of 0.1 M sodium citrate). A BMG labtech Fluostar Omega Microplate reader was then used to measure the luminescence intensity at 25°C immediately after the addition of the D-luciferin.

RESULTS AND DISCUSSIONS

Antimicrobial susceptibility testing

The antimicrobial susceptibilities of test compounds (TT225 and nitroxoline derivatives) were determined by comparing them with established novel antibacterial agents (which represented the control agents for mode of action analysis). MIC values for the control agents tetracycline and fosfomycin were lower against *S. aureus* than *B. subtilis*. However, for vancomycin and D- cycloserine, the opposite was observed, with MIC values of 0.5 µg/ml and 32 µg/ml against *B. subtilis* compared to 4 and 64 µg/ml against *S. aureus*. Flucloxacillin had the lowest MIC value among the control agents at 0.125 µg/ml against both microorganisms. MIC values for CTAB were the same for both microorganisms at 2 µg/ml concentration each. MICs value for triclosan and rifampicin were determined only against *B. subtilis* as the MICs value was used for the *B. subtilis* biosensor.

For TT225, the MIC against *S. aureus* was 256 µg/ml. The antibacterial activities of the nitroxoline derivatives differed between the two microorganisms tested. Nitroxoline itself, as the parent of the derivatives, displayed a MICs value of 2µg/ml against *B. subtilis* compared to 8 µg/ml for *S. aureus*. From 27 derivatives, only 2 compounds (GIS213 and GISB4) showed inhibition activity ≤ 256 µg/ml against *S. aureus* whereas in case of *B. subtilis*, 13 compounds showed MICs of ≤ 256 µg/ml. The data were tabulated in Table 2 and Table 3. The study showed that TT225, nitroxoline and its derivatives have potential to be antibacterial agents. (GISB2, GISB4, GISB5, GISB6, GISB7, GISB13, GIS213, GIS241_P, GIS244, GIS249_2, GJ19, GJ21 and GJ22.2), versus 2 compounds (GISB4 AND GIS213) were active against *S. aureus*. The inhibition is much more active against *B. subtilis* compared to *S. aureus*. This was observed in the nitroxoline (control agent for nitroxoline derivatives) exhibit better antibacterial activity against *B. subtilis* in compared to *S. aureus*. The factor that may contribute to the findings could be the structure of the organisms. Both microorganisms are Gram-positive bacteria. Among the Gram-positive bacteria itself, there are many differences present. One of the differences relates to the peptide crosslinks between glycan strands [10]. *S. aureus* presence branched stem peptides that are involved in the crosslinking while *B. subtilis* does not ([11]. The major role of the branched stem peptide is preparing attachment sites for the covalently associated proteins [9]. This could be one of the factors that contributes to the difference in the antibacterial activity of the compounds towards the microorganisms.

Table 1: MICs determination of established antibiotics against *S. aureus* and *B. subtilis*

Drug	<i>S. aureus</i> SH1000 MIC ($\mu\text{g/ml}$)	<i>B. subtilis</i> 1S34 MIC ($\mu\text{g/ml}$)
Vancomycin	4	0.5
Tetracycline	1	16
D-cycloserine	64	32
Fosfomycin	8	128
Flucloxacillin	0.125	0.125
CTAB	2	2
Ciprofloxacin	2	0.125
8-hydroxyquinoline	2	2
Triclosan	ND	0.625
Rifampicin	ND	128

Table 2: MIC ($\mu\text{g/mL}$) of TT225 and nitroxoline derivatives against *S.aureus*

Antibacterial agent	<i>S. aureus</i> SH1000 MIC ($\mu\text{g/ml}$)
TT225	256
Nitroxoline	8
GIS213	64
GISB4	256

Table 3: MIC ($\mu\text{g/mL}$) of TT225 and nitroxoline derivatives against *B.subtilis*

Antibacterial agent	<i>B. subtilis</i> 1S34 MIC ($\mu\text{g/ml}$)
TT225	ND
Nitroxoline	2
GIS213	64
GISB4	64
GISB2	128
GISB5	128
GISB6	128
GISB13	64
GIS241_P	64
GIS244	256
GIS249_2	256
G.J-19	64
G.J-21	128
G.J-22.2	256

B.subtilis antibiotic biosensors for mode of action identification

5 cellular biosensors (DNA, RNA, protein, fatty acid and cell envelope) were utilised in this study in order to identify the mode of action of the novel agent *B. subtilis* is often used as a model organism in molecular and cellular biology, as it has the ability to produce measurable regulatory responses to different type of extracellular stress [10]. The induction values for each of the biomarkers are 2.5 fold for *yorB*, 2 fold for *yvgS*, *yheI* and *fabHB* and 1.7 fold for *ypuA* [12]. The test compounds were chosen to undergo the assay on the basis of MICs values that are $\leq 256 \mu\text{g/ml}$. 13 compounds representing nitroxoline and its derivatives were validated in the biosensor assay as shown in Table 4. Unfortunately, the nitroxolines did not induce induction of any of the biosensors, with the exception of GIS249_2 and GISB4, which both induce *yorB* (DNA synthesis) with above threshold with (5.2 ± 0.2 and 3.5 ± 0.3 respectively). The nitroxoline compounds were expected to induce the biosensor responsive to DNA synthesis due to their structural similarity to 8-hydroxyquinoline, a DNA synthesis inhibitor which has been previously shown to induce *yorB* [8]. The published induction ratio was 4.7 ± 1.1 , and this used to compare with the induction of nitroxoline and its derivatives. The structure of GIS249_2 that possesses a primary amine group while GISB4 had an extra N-functional group linked to the methyl (CH_3) which is absence from the other nitroxoline derivatives. This can be the factor that contributes to the induction of signal in the biosensor. As such, this functional group may be crucial for the inhibitory activity of these compounds on DNA synthesis.

Table 4: Induction of *Bacillus subtilis* antibiotic biosensors in response to test and control antibacterial agents

Antibacterial Agent	Upregulated biosensor promoter				
	Cell-envelope (<i>ypuA</i>)	Protein (<i>yheI</i>)	RNA (<i>yvgS</i>)	DNA (<i>yorB</i>)	Fatty-acid (<i>fabHB</i>)
Nitroxoline	- (1.4 ±0.1)	- (0.7 ±0.4)	- (1.2 ±0.3)	- (0.7 ±0.3)	- (1.6 ±0.3)
GISB2	- (0.8 ±0.1)	- (0.5 ±0.3)	- (1.4 ±0.2)	- (0.8 ±0.2)	- (0.9 ±0.1)
GISB5	- (1.2 ±0.1)	- 0.8 ±0.5)	- (1.4 ±0.3)	- 0.8 ±0.3)	- (0.9 ±0.1)
GISB6	- (1.0 ±0.1)	- (0.7 ±0.4)	- (1.1 ±0.1)	- (0.9 ±0.4)	- (1.5 ±0.8)
GISB4	- (1.0 ±0.4)	- (0.7 ±0.1)	- (1.9 ±0.4)	+ (3.5 ±0.3)	- (1.3 ±0.1)
GISB7	- (1.0 ±0.2)	- (0.7 ±0.3)	- (1.3 ±0.3)	- (0.8 ±0.2)	- (1.2 ±0.4)
GISB13	- (1.2 ±0.4)	- (0.9 ±0.6)	- (1.0 ±0.1)	- (1.0 ±0.3)	- (1.2 ±0.7)
GJ-19	- (1.3 ±0.5)	- (0.6 ±0.3)	- (1.4 ±0.1)	- (0.9 ±0.3)	- (1.4 ±0.5)
GJ-21	- (1.0 ±0.4)	- (0.8 ±0.2)	- (1.7 ±0.5)	- (0.7 ±0.4)	- (1.1 ±0.5)
GJ-22.2	- (0.9 ±0.3)	- (0.7 ±0.2)	- (1.6 ±0.2)	- (0.7 ±0.3)	- (1.0 ±0.2)
GIS249_2	- (1.2 ±0.4)	- (0.7 ±0.2)	- (1.8 ±0.1)	+ (5.2 ±0.2)	- (1.5 ±0.1)
GIS241_P	ND	- (0.9 ±0.2)	- (1.6 ±0.2)	- (0.8 ±0.2)	- (1.7 ±0.3)
GIS244	ND	- (1.3 ±0.1)	- (1.7 ±0.2)	- (0.7 ±0.4)	- (1.3 ±0.3)

CONCLUSIONS

Among 27 compounds provided for the study, only 3 compounds (TT225 and GIS213, GISB4 (nitroxoline derivatives) showed MICs value that is $\leq 256\mu\text{g/ml}$ against *S. aureus*. The compounds were then tested for the mode of action. The nitroxoline derivatives were analysed for the mode of action according to displaying activity against *B. subtilis*. Even though these compounds are structurally similar to 8-hydroxyquinoline which was previously been shown to induce *yorB* [8, 12], only two of the compounds showed similar activity, and inhibition of DNA synthesis. By using these biosensors, it has enabled us to provide a novel insight regarding the mode of action of these antibacterial agents. In addition as these compounds show specific inhibition of DNA synthesis, they may be taken for further development as potential chemotherapeutic candidates.

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