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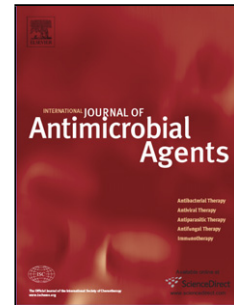
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## Antimicrobial activities of trimethoprim/sulfamethoxazole, 5-iodo-2'-deoxyuridine and rifampicin against *Staphylococcus aureus*

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## ABSTRACT

Trimethoprim/sulfamethoxazole (SXT), alone and in combination with rifampicin (RIF), is a therapeutic option against *Staphylococcus aureus*, including strains expressing methicillin resistance. However, the antimicrobial activity of SXT is antagonised by thymidine, which can be present in infected and/or inflamed tissues such as the airways of cystic fibrosis (CF) patients. In this study, thymidine concentrations in CF sputa were determined and the antimicrobial activities of SXT, 5-iodo-2'-deoxyuridine (IdUrd) and RIF alone and in combination against *S. aureus* were analysed. Thymidine concentrations in the sputa of ten different CF patients varied from <100 µg/L to 38 845 µg/L. The abolished antimicrobial activity of SXT against 22 *S. aureus* strains in the presence of thymidine was restored by combination with IdUrd. In contrast, SXT combined with RIF in the presence of thymidine did not show a synergistic effect and, furthermore, allowed the emergence of RIF-resistant bacteria. Adding RIF to the combination of SXT and IdUrd did not improve antimicrobial activity against *S. aureus*. In conclusion, the combination of SXT and RIF as a therapeutic option against *S. aureus* infections in chronic inflamed tissues should be judged critically.

## 1. Introduction

*Staphylococcus aureus* is a major human pathogen and is one of the most common bacteria isolated from the airways of cystic fibrosis (CF) patients [1]. Moreover, an increase in the prevalence of methicillin-resistant *S. aureus* (MRSA) has been noted in CF patients in the USA [1]. A treatment option against *S. aureus* in general and MRSA in particular is trimethoprim/sulfamethoxazole (SXT) either alone [1] or in combination with rifampicin (RIF) [2].

Trimethoprim and sulfamethoxazole inhibit different enzymatic steps of the folic acid pathway, leading to cessation of bacterial synthesis of thymidine monophosphate (dTMP) via thymidylate synthase [3,4]. However, the antimicrobial activity of folic acid antagonists (FAAs) such as SXT can be antagonised by bacterial utilisation of thymidine. Various bacteria have the ability to use an alternative pathway by uptake of extracellular thymidine and subsequent intracellular phosphorylation to dTMP by thymidine kinase. Thymidine is expected to be abundant in the airway secretions of CF patients owing to the presence of necrotic cells that release DNA, which in turn can be catabolised via dTMP to thymidine [4]. Failure rates of SXT therapy are high, in particular in the presence of necrotic cells [3]. Recently, we demonstrated that the in vitro combination of SXT and a nucleoside analogue showed significantly improved antimicrobial activity against *S. aureus* in the presence of thymidine because nucleoside analogues such as 5-iodo-2'-deoxyuridine (IdUrd) inhibit bacterial thymidine utilisation [4].

RIF should not be used as monotherapy against *S. aureus* because of the danger of the emergence of resistance [5]. There are various studies with different results

regarding the combined antimicrobial activity of FAAs and RIF against staphylococci [6,7]. However, to the best of our knowledge, there are no data regarding the combined antimicrobial activity of SXT and RIF against *S. aureus* in the presence of thymidine.

The aim of this study was to determine thymidine concentrations in the sputa of CF patients and to analyse the antimicrobial activity of SXT, IdUrd and RIF alone and in combination against *S. aureus* in the presence of thymidine.

## 2. Materials and methods

### 2.1. High-performance liquid chromatography (HPLC)

Sputa from 10 different CF patients attending the University Hospital of Frankfurt am Main (Frankfurt am Main, Germany) were analysed. To determine thymidine concentrations, extraction of nucleosides was performed and samples were separated by HPLC as described previously [8].

### 2.2. Bacterial strains

Twelve methicillin-susceptible *S. aureus* (MSSA) (ATCC 29213, ATCC 25923 and 10 non-duplicate clinical blood culture isolates from the University Hospital of Frankfurt am Main) as well as ten MRSA (ATCC 43300, and MRSA of multilocus sequence types ST29, ST22, ST45, ST228, ST5, ST1, ST8, ST80 and ST152) were used in this study. All *S. aureus* isolates were SXT- and RIF-susceptible.

### 2.3. Antimicrobial susceptibility testing

#### 2.3.1. Minimum inhibitory concentrations (MICs)

The MIC was defined as the lowest concentration of antimicrobial agent that completely inhibited growth of the organism in a microdilution well as detected by the unaided eye [9]. Growth inhibition as detected by the unaided eye was confirmed by measuring the turbidity of wells with a microplate reader.

The MICs of RIF and SXT ± IdUrd (100 µmol/L) against *S. aureus* with and without addition of thymidine (2000 µg/L) were determined in microtitre plates following Clinical and Laboratory Standards Institute (CLSI) guidelines [9]. This thymidine concentration was chosen as it exists in human CF sputa.

#### 2.3.2. Fractional inhibitory concentration index (FICI)

The antimicrobial activity of SXT in combination with RIF with and without supplementation of thymidine (2000 µg/L) and IdUrd (100 µmol/L) was assessed against 22 *S. aureus* strains by chequerboard assay using commercial plates (Merlin, Rüsselsheim, Germany). SXT was tested at concentrations of 0.078–80 mg/L at a ratio of 1:19, i.e. trimethoprim 0.0039–4 mg/L and sulfamethoxazole 0.074–76 mg/L. RIF was tested at concentrations of 0.001–0.064 mg/L. FICIs of ≤0.5 were interpreted as synergistic, FICIs of >0.5 to 4.0 were interpreted as indifferent and FICIs of >4 were interpreted as antagonistic.



### 2.3.3. Time–kill studies

The antimicrobial activity of SXT, IdUrd and RIF alone and in combination against *S. aureus* ATCC 29213 was determined by the time–kill method as described previously [4] following CLSI guidelines [9,10]. Bacterial suspensions supplemented with SXT (0.5× and 4× MIC, i.e. 0.25 mg/L and 2 mg/L, respectively), IdUrd (10 µmol/L), RIF (0.5× and 4× MIC, i.e. 0.004 mg/L and 0.032 mg/L, respectively) and thymidine (200 µg/L) were incubated for various time intervals (0, 2, 4, 6 and 24 h). As the thymidine concentration used in the checkerboard assay (2000 µg/L) was above the median of the thymidine concentrations found in CF sputa, and in order to use a second relevant thymidine concentration in this study, a thymidine concentration below the median of the thymidine concentrations found in CF sputa (i.e. 200 µg/L) was chosen for the time–kill assay. The activity of SXT and RIF was verified by MIC determination against the reference strain *S. aureus* ATCC 29213 [9]. Drug carryover was excluded as previously described [4]. Experiments were performed in triplicate. Synergy was defined as a  $\geq 2 \log_{10}$  decrease in colony-forming units (CFU)/mL between the combination and its most active constituent after 24 h, with the less active component being tested at an ineffective concentration [10]. Indifference was defined as  $< 2 \log_{10}$  difference and antagonism as  $\geq 2 \log_{10}$  increase between the CFU/mL of the combination compared with the CFU/mL of the most potent single drug. Bacteriostatic activity was defined as a 0 to  $< 3 \log$  killing and bactericidal activity as a  $\geq 3 \log$  killing after 24 h.

### 2.4. Selection of rifampicin-resistant mutants

*Staphylococcus aureus* ATCC 29213 was cultured overnight in cation-adjusted Mueller–Hinton broth (CA-MHB). The culture was then concentrated 100:1 by

centrifugation (5000 rpm, 10 min) and was subsequently re-suspended in CA-MHB. Then, 100  $\mu$ L of various dilutions (range 1:1–1:10<sup>7</sup>) of this concentrated suspension was streaked on cation-adjusted Mueller–Hinton agar (CA-MHA) supplemented with different drugs, i.e. RIF (1 mg/L), SXT (40 mg/L), thymidine (2000 mg/L) and IdUrd (100  $\mu$ mol/L), in different combinations. CFUs on the different agar plates were enumerated after 72 h of incubation at 37 °C. The frequency of mutations to RIF resistance in *S. aureus* was evaluated by dividing the number of RIF-resistant bacteria/mL by the bacterial inoculum/mL. Colonies were analysed for RIF and SXT resistance by RIF and SXT Etests on CA-MHA following the manufacturer's guidelines. Experiments were performed in triplicate.

### 3. Results

#### 3.1. Thymidine concentrations in cystic fibrosis sputa

The thymidine concentration in sputa of 10 different CF patients was measured by HPLC. Concentrations were 394, 668, 796, 3127, 11 976 and 38 845  $\mu$ g/L; in four CF sputa the thymidine concentration was below the limit of detection (thymidine concentration ca. <100  $\mu$ g/L). The median concentration was 531  $\mu$ g/L.

#### 3.2. Antimicrobial activity of trimethoprim/sulfamethoxazole in combination with 5-iodo-2'-deoxyuridine against *Staphylococcus aureus* in the presence of thymidine

Time–kill kinetics of SXT (0.5 $\times$  and 4 $\times$  MIC) and IdUrd (10  $\mu$ mol/L) in the presence of thymidine (200  $\mu$ g/L) against *S. aureus* ATCC 29213 was determined.

*Staphylococcus aureus* without the addition of any antibiotic showed 2.76 log growth within 24 h. IdUrd alone did not show any antimicrobial activity [ $\log_{10}$  (CFU<sub>24 h</sub>/CFU<sub>0 h</sub>)

= 3.039] after 24 h of incubation at 37 °C. SXT at 4× MIC alone and SXT at 0.5× MIC alone or in combination with IdUrd (10 µmol/L) also allowed bacterial growth, with  $\log_{10}$  (CFU<sub>24 h</sub>/CFU<sub>0 h</sub>) values ranging from 2.40 to 2.73. In contrast, SXT at 4× MIC in combination with IdUrd (10 µmol/L) showed synergistic antimicrobial activity with a bactericidal effect [ $\log_{10}$  (CFU<sub>24 h</sub>/CFU<sub>0 h</sub>) = -3.04].

Moreover, the MICs of SXT with and without supplementation of IdUrd and thymidine were analysed against 22 *S. aureus* strains. Without addition of thymidine, MICs of SXT ranged from 0.63 mg/L to 2.5 mg/L. In the presence of thymidine (2000 µg/L), MICs of SXT against 20 strains were >80 mg/L. In contrast, antimicrobial activity of SXT in the presence of thymidine was restored by combination with IdUrd (MICs ranging from 0.63 mg/L to 2.5 mg/L). These results show that the antimicrobial activity of SXT against different *S. aureus* strains is reduced by thymidine but is restored by combination with IdUrd.

### 3.3. Antimicrobial activity of trimethoprim/sulfamethoxazole in combination with rifampicin against *Staphylococcus aureus* ATCC 29213 in the presence of thymidine

Time–kill kinetics revealed that in the presence of thymidine (200 µg/L), SXT (0.5× and 4× MIC) alone or combined with a subinhibitory concentration of RIF (0.5× MIC) allowed bacterial growth, with  $\log_{10}$  (CFU<sub>24 h</sub>/CFU<sub>0 h</sub>) values ranging from 1.48 to 2.66 (Fig. 1). RIF alone at 4× MIC showed bacteriostatic antimicrobial activity, with a  $\log_{10}$  (CFU<sub>24 h</sub>/CFU<sub>0 h</sub>) value of -0.81; addition of SXT (0.5× or 4× MIC) in the presence of thymidine showed an indifferent effect, with  $\log_{10}$  (CFU<sub>24 h</sub>/CFU<sub>0 h</sub>) values of -0.47 and -1.15, respectively.

Moreover, the chequerboard method revealed that without addition of thymidine, the antimicrobial activity of the combination of SXT and RIF against different *S. aureus* strains, including MRSA, was indifferent, with FICIs ranging from 0.50 to 1.50 (Table 1). In the presence of thymidine, no FICIs could be calculated, as in general no MICs of SXT could be determined. Therefore, minimum effective concentrations (MECs) of SXT in the presence of thymidine were determined as described previously [11]. The antimicrobial activity of RIF against all *S. aureus* strains tested was not or was not substantially improved by combination with SXT in the presence of thymidine (data not shown). These data demonstrate that combination with SXT did not substantially improve the antimicrobial activity of RIF against *S. aureus* in the presence of thymidine.

#### *3.4. Antimicrobial activity of trimethoprim/sulfamethoxazole plus 5-iodo-2'-deoxyuridine in combination with rifampicin against Staphylococcus aureus in the presence of thymidine*

Time–kill assays were performed to analyse the antimicrobial activity of SXT plus IdUrd alone and in combination with RIF against *S. aureus* ATCC 29213 in the presence of thymidine. SXT (0.5× MIC) plus IdUrd (10 µmol/L) with or without RIF (0.5× MIC) did not show any antimicrobial activity (Fig. 2). Bacteriostatic activity was observed for RIF at a high concentration (4× MIC) alone or in combination with SXT (0.5× MIC) plus IdUrd (10 µmol/L). In contrast, the antimicrobial combination of SXT (4× MIC) and IdUrd (10 µmol/L) showed bactericidal activity, with  $\log_{10}$  (CFU<sub>24 h</sub>/CFU<sub>0 h</sub>) values of –3.04 in the presence of thymidine. However, antimicrobial activity was reduced by combination with RIF (0.5× and 4× MIC), showing  $\log_{10}$  (CFU<sub>24 h</sub>/CFU<sub>0 h</sub>) values of –1.73 (indifference) and –0.80 (antagonism), respectively.

Moreover, the checkerboard method revealed that in the presence of thymidine (2000 µg/L) and IdUrd (100 µmol/L), indifference between SXT and RIF (FICIs between 0.50 and 1.50) against 22 *S. aureus* strains could be observed (Table 1). This argues against a triple therapy consisting of SXT, IdUrd and RIF since there is no additional benefit by combination with RIF.

### 3.5. Selection of rifampicin-resistant mutants *in vitro*

The frequency of mutations conferring RIF resistance in *S. aureus* in the presence of RIF, SXT, thymidine and IdUrd was evaluated. Whilst mutation rates in the presence of RIF alone were high ( $2 \times 10^{-8}$ ), no RIF-resistant bacteria could be detected when SXT was added (i.e. RIF + SXT). However, by additional supplementation of thymidine (i.e. RIF + SXT + thymidine), a similar mutation rate was found ( $8 \times 10^{-9}$ ). Mutants were resistant to RIF but not to SXT, as shown by Etest (data not shown). Selection of RIF-resistant bacteria could be totally inhibited by additional supplementation of IdUrd (i.e. RIF + SXT + thymidine + IdUrd).

## 4. Discussion

Thymidine is expected to be abundant in damaged host tissues and airway secretions of CF patients [3,8]. In this study, ten different CF sputa were analysed to confirm that thymidine concentrations in CF sputa are elevated. Thymidine concentrations ranged from <100 µg/L to 38 845 µg/L, which emphasises the importance of thymidine in CF sputa as an antagonist of SXT.

SXT is proposed as an alternative antibiotic against *S. aureus* infections in CF patients [1]. Moreover, SXT has also been suggested as an oral antibiotic against multiresistant MRSA [5]. In our opinion, therapy with SXT against *S. aureus* affecting the airways of CF patients should be judged critically, because failure of SXT therapy may occur due to thymidine released from damaged host tissues [3]. Indeed, *S. aureus* continued to grow in the presence of SXT when thymidine was supplemented at concentrations that exist in sputa of CF patients. Moreover, thymidine-dependent small-colony variants have been shown to emerge particularly following long-term SXT treatment in CF patients and are described to be associated with more advanced disease [12].

Several authors suggest that application of SXT or trimethoprim each in combination with RIF could be an alternative antimicrobial therapy against *S. aureus*, in particular MRSA [2,5]. There are different studies regarding the combined antimicrobial activity of FAAs and RIF against *S. aureus*. Some authors found a trend towards antagonism, whereas others observed partial synergism between FAAs and RIF [6,7]. In several studies, the combination of RIF and trimethoprim prevented the emergence of bacteria resistant to RIF or trimethoprim, which were often seen when one agent was used alone [13,14].

These observations present an argument for the combination of FAAs and RIF. In this study, it was shown that combination of RIF and SXT did not show synergistic effects and failed to prevent the emergence of RIF resistance in *S. aureus* in the presence of elevated thymidine concentrations. In our opinion, infections involving necrotic tissues should therefore not be treated with combination therapy consisting of FAAs and RIF.

Recently, we screened for nucleoside analogues such as IdUrd that impair bacterial thymidine utilisation [4]. In the presence of thymidine, IdUrd, a thymidine kinase inhibitor, significantly improved the antimicrobial activity of SXT against different bacterial species [4]. IdUrd has already been tested in phase I trials [15]. Dose-limiting toxicity for IdUrd was determined at steady-state plasma concentrations of ca. 100  $\mu\text{mol/L}$ . This study shows that in the presence of thymidine, combination of SXT with IdUrd improved the antimicrobial activity of SXT against all *S. aureus* strains tested. Additional supplementation of RIF did not further improve this antibacterial effect.

In conclusion, these data suggest that SXT in combination with RIF may fail to cure *S. aureus* infections in tissues with elevated thymidine concentrations. However, SXT in combination with a thymidine kinase inhibitor may become an interesting antimicrobial combination for this setting in the future. Further in vitro and in vivo studies are therefore required.

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**Competing interests**

None declared.

**Ethical approval**

Not required.

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**Fig. 1.** Time–kill curves showing the combined antimicrobial activity of trimethoprim/sulfamethoxazole (SXT) (0.5× and 4× MIC) and rifampicin (RIF) (0.5× and 4× MIC) alone and in combination at a constant thymidine concentration (200 µg/L) against *Staphylococcus aureus* ATCC 29213. Mean values from three different experiments are shown. Error bars indicate standard deviations. MIC, minimum inhibitory concentration. CFU, colony-forming units.

**Fig. 2.** Time–kill curves showing the antimicrobial activity of trimethoprim/sulfamethoxazole (SXT) (0.5× and 4× MIC) plus 5-iodo-2'-deoxyuridine (IdUrd) (10 µmol/L) alone and in combination with rifampicin (RIF) (0.5× and 4× MIC) at a constant thymidine concentration (200 µg/L) against *Staphylococcus aureus* ATCC 29213. Mean values from three different experiments are shown. Error bars indicate standard deviations. MIC, minimum inhibitory concentration. CFU, colony-forming units.

**Table 1**

Fractional inhibitory concentration indices (FICIs) of trimethoprim/sulfamethoxazole (SXT) and rifampicin (RIF) with and without supplementation of thymidine (2000  $\mu\text{g/L}$ ) and 5-iodo-2'-deoxyuridine (IdUrd) (100  $\mu\text{mol/L}$ )

Strain	FICI [median (range)]	
	SXT + RIF	SXT + RIF in the presence of thymidine and IdUrd
MRSA ATCC 43300	0.59 (0.50–1.03)	0.91 (0.75–1.25)
MRSA ST29	1.25 (1.06–1.50)	1.25 (1.06–1.50)
MRSA ST22	1.03 (0.63–1.29)	1.13 (1.06–1.25)
MRSA ST45	1.25 (1.03–1.50)	1.19 (1.06–1.50)
MRSA ST228	1.03 (0.63–1.25)	1.15 (1.00–1.25)
MRSA ST5	1.25 (1.00–1.25)	1.25 (1.00–1.25)
MRSA ST1	1.06 (0.76–1.29)	1.19 (1.06–1.50)
MRSA ST8	1.13 (1.00–1.25)	1.13 (1.00–1.25)
MRSA ST80	1.19 (1.06–1.50)	1.13 (1.00–1.25)
MRSA ST152	1.13 (1.00–1.25)	1.06 (0.75–1.13)
MSSA ATCC 29213	1.25 (1.06–1.50)	1.06 (0.56–1.25)
MSSA ATCC 25923	1.13 (1.03–1.50)	1.13 (0.50–1.50)

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MSSA V7/31528	1.06 (0.75– 1.25)	1.00 (0.56–1.25)
MSSA V7/34062	1.00 (0.56– 1.25)	1.13 (1.00–1.25)
MSSA V7/34124	1.06 (0.75– 1.25)	1.19 (1.06–1.50)
MSSA V7/43688	0.75 (0.53– 1.13)	1.06 (0.75–1.25)
MSSA V8/22501	1.03 (0.63– 1.13)	1.25 (1.06–1.50)
MSSA V8/51379	1.06 (0.75– 1.25)	1.13 (0.75–1.50)
MSSA V9/15348	1.06 (0.63– 1.25)	1.05 (0.63–1.25)
MSSA V9/18889	1.13 (1.00– 1.25)	1.04 (0.63–1.25)
MSSA V9/66121	0.63 (0.53– 0.75)	1.06 (0.75–1.25)
MSSA V9/66800	1.25 (1.06– 1.50)	1.06 (0.75–1.13)

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MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-susceptible *S. aureus*.

Figure 1.

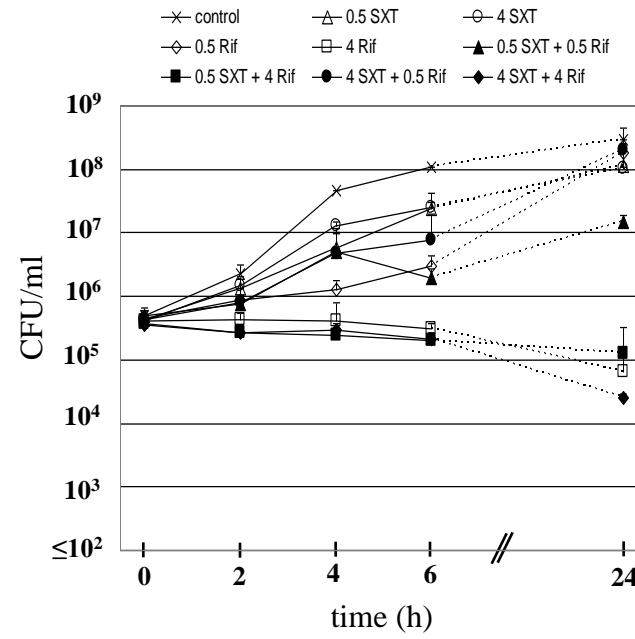


Figure 2.

