International Journal of Antimicrobial Agents 36 (2010) 562–565

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: http://www.elsevier.com/locate/ijantimicag

Short communication

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

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ARTICLE INFO

Article history:
Received 26 March 2010
Accepted 11 August 2010

Keywords:
Rifampicin
Trimethoprim/sulfamethoxazole
5-Iodo-2′-deoxyuridine
Antimicrobial activity
Thymidine

ABSTRACT

Trimethoprim/sulfamethoxazole (SXT), alone and in combination with rifampicin (RIF), is a therapeutic option against *Staphylococcus aureus*, including strains expressing meticillin 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 meticillin-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

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doi:10.1016/j.ijantimicag.2010.08.007

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 meticillin-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 chequerboard 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 ≥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 μL of various dilutions (range 1:1–1:10^7) 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 μmol/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 μg/L; in four CF sputa the thymidine concentration was below the limit of detection (thymidine concentration ca. <100 μg/L). The median concentration was 531 μg/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× and 4× MIC) and IdUrd (10 μmol/L) in the presence of thymidine (200 μg/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 [$\log_{10}(CFU_{24\,h}/CFU_{0\,h}) = 3.039$] 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 $\log_{10}(CFU_{24\,h}/CFU_{0\,h})$ 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 [$\log_{10}(CFU_{24\,h}/CFU_{0\,h}) = -3.04$].

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 $\log_{10}(CFU_{24\,h}/CFU_{0\,h})$ values ranging from 1.48 to 2.66 (Fig. 1). RIF alone at 4× MIC showed bacteriostatic antimicrobial activity, with a $\log_{10}(CFU_{24\,h}/CFU_{0\,h})$ value of −0.81; addition of SXT