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„Antimicrobial effects of antiplatelet drugs and antimicrobial / antiplatelet drug combinations"

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Abstract

Dual antiplatelet therapy (DAPT) consisting of a $P2Y_{12}$ receptor inhibitor and acetylsalicylic acid (aspirin) plays a fundamental role in the pharmacological management of acute coronary syndrome (ACS). Currently, there is a growing body of evidence to antimicrobial activity of the $P2Y_{12}$ inhibitor ticagrelor, but some limitations in previous studies arose due to small numbers of tested bacteria. In order to expand the knowledge about antimicrobial properties of ticagrelor and also other cardiovascular drugs used in cardiovascular diseases, we performed in-vitro antimicrobial susceptibility testing including a larger number of bacterial strains. Another aim of the present study was to investigate combinational effects between salicylic acid, the major metabolite of aspirin, and ticagrelor, as well as between different antibiotics and ticagrelor. The methodology used for these purposes was agar-dilution for screening antimicrobial activity, broth-microdilution to determine minimum inhibitory concentrations (MICs) of ticagrelor and a combined method consisting of the Epsilometertest-method and agar dilution to perform a synergy-screening between antiplatelet drugs and antibiotics. This work describes antimicrobial activity of ticagrelor against 28 gram-positive bacterial strains, that include drug-resistant bacteria knowing to cause severe infections like endocarditis. Moreover, we provide data for an amplifying antimicrobial activity between ticagrelor in combination with salicylic acid and the results evaluated by the antibiotic/antiplatelet synergy screening may suggest that ticagrelor has the capacity to enhance antibacterial activity of some antibiotics.

Deutsche Zusammenfassung

Duale Antiplättchen-Therapie (DAPT), bestehend aus einem $P2Y_{12}$-Rezeptor-Inhibitor und Acetylsalicylsäure (Aspirin), stellt den Eckpfeiler der pharmakologischen Behandlung des akuten Koronarsyndroms (ACS) dar. Gegenwärtig gibt es eine wachsende Zahl von Belegen für die antimikrobielle Aktivität von Ticagrelor, einem $P2Y_{12}$-Inhibitor, aber es ergaben sich einige Einschränkungen in früheren Studien aufgrund der geringen Anzahl getesteter Bakterien. Um das Wissen über die antimikrobiellen Eigenschaften von Ticagrelor und auch anderen kardiovaskulären Medikamenten zu erweitern, führten wir in-vitro antimikrobielle Empfindlichkeitstests an einer großen Anzahl von Bakterienstämmen durch. Ein weiteres Ziel der vorliegenden Studie war die Untersuchung von Kombinationswirkungen zwischen Salicylsäure, dem Hauptmetaboliten von Aspirin, und Ticagrelor sowie zwischen Antibiotika und Ticagrelor. Die dafür verwendete Methodik war die Agar-Dilution zum Screening der antimikrobiellen Aktivität, Mikrodilution zur Bestimmung der minimalen Hemmkonzentrationen (MHK) von Ticagrelor und eine kombinierte Methode, bestehend aus der Epsilometertest-Methode und der Agar-Dilution, um ein Synergie-Screening zwischen Thrombozytenaggregationshemmern und Antibiotika durchzuführen. Diese Arbeit beschreibt die antimikrobielle Aktivität von Ticagrelor gegen 28 gram-positive Bakterienstämme, zu denen auch arzneimittelresistente Bakterien gehören, von denen bekannt ist, dass sie schwere Infektionen wie Endokarditis verursachen. Darüber hinaus liefern wir Daten über eine verstärkende antimikrobielle Aktivität zwischen Ticagrelor in Kombination mit Salicylsäure und die Ergebnisse, die durch das Antibiotika/Anti-Plättchen-Synergie-Screening ausgewertet wurden, deuten darauf hin, dass Ticagrelor die Fähigkeit hat, die antibakterielle Aktivität einiger Antibiotika zu verstärken.

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Abbreviations

ACS	acute coronary syndrome
ASA	acetylsalicylic acid
ATCC	American Type Culture Collection
C_{max}	maximum plasma concentration
CoNS	coagulase negative staphylococci
DAPT	dual antiplatelet therapy
DMSO	dimethyl sulfoxide
DSMZ	German Collection of Microorganisms and Cell Cultures GmbH
E-test	Epsilometertest
E. coli	Escherichia coli
E. faecalis	Enterococcus faecalis
E. faecium	Enterococcus faecium
EUCSAT	European Committee on Antimicrobial Susceptibility Testing
IE	infective endocarditis
MHA	Mueller-Hinton agar
MHB	Mueller-Hinton broth
MI	myocardial infarction
MICs	minimum inhibitory concentrations
MRSA	methicillin-resistant Staphylococcus aureus
MRSE	methicillin-resistant Staphylococcus epidermidis
MSSA	methicillin-sensitive Staphylococcus aureus
NaCl	sodium chloride
S.	Staphylococcus / Staphylococci
SA	salicylic acid
STEMI	ST-element elevation myocardial infarction
VRE	vancomycin-resistant Enterococci

1. Introduction

Cardiovascular diseases represent the most common cause of death worldwide and 85% of these deaths are attributable to myocardial infarction (MI) and stroke (WHO, 2017).

One important subcategory of cardiovascular diseases is acute coronary syndrome (ACS), a clinical term, which conflates the occurrence of MI and unstable angina pectoris (Sanchis-Gomar et al., 2016). Depending on electrocardiographic presentation, MI can be separated into ST-segment elevation myocardial infarction (STEMI) and non-STEMI (Gach et al., 2018). Antiplatelet drugs and dual antiplatelet therapy (DAPT) play a pivotal role in the pharmacological management of ACS. Referring to recent guidelines, DAPT consisting of acetylsalicylic acid (ASA) in combination with a $P2Y_{12}$ receptor inhibitor (ticagrelor or prasugrel), taken for one year, presents the standard pharmacological treatment in patients with non-STEMI-ACS (Collet et al., 2020). For patients who underwent percutaneous coronary intervention after STEMI, DAPT for one year is recommended (Ibanez et al., 2017).

In a post-hoc analysis of the PLATelet inhibition and patients Outcomes (PLATO) study of patients with ACS, Storey et al. (2014) demonstrated that the number of deaths attributable to sepsis or pulmonary adverse events was significantly lower in the group of patients who received ticagrelor in contrast to the clopidogrel group (Storey et al., 2014). In another study, the investigators suggested superiority of ticagrelor, when compared to clopidogrel, in patients after a STEMI suffering from methicillin-sensitive *Staphylococcus aureus* (MSSA) or methicillin-resistant *S. aureus* (MRSA) infections (Rigatelli et al., 2019). More recently, a significantly decreased risk of gram-positive infections in patients with ACS, receiving DAPT consisting of ASA and ticagrelor compared to ASA and clopidogrel, was shown (Lupu et al., 2020).

1.1. Ticagrelor

Ticagrelor is an antiplatelet drug for oral administration, that directly binds and reversibly antagonizes the $P2Y_{12}$ receptor (Nylander et al., 2013). To exert its pharmacological effects, there is no need for metabolic activation of ticagrelor (Husted et al., 2006). Taken concomitantly with ASA, ticagrelor is approved to prevent atherothrombotic events in patients with ACS, as well as in patients with a previous MI who are at high risk of developing an atherothrombotic event (European Medicines Agency, 2016). Beyond its antimicrobial

properties, previous studies showed immunomodulating activities of ticagrelor (Jiang et al., 2018, Sexton et al., 2018). The XANTHIPPE trial performed by Sexton et al. (2018) demonstrated a significant decrease of the pro-inflammatory cytokine interleukin-6 in patients with pneumonia receiving ticagrelor compared to placebo (Sexton et al., 2018).

Figure 1. Chemical structure of ticagrelor
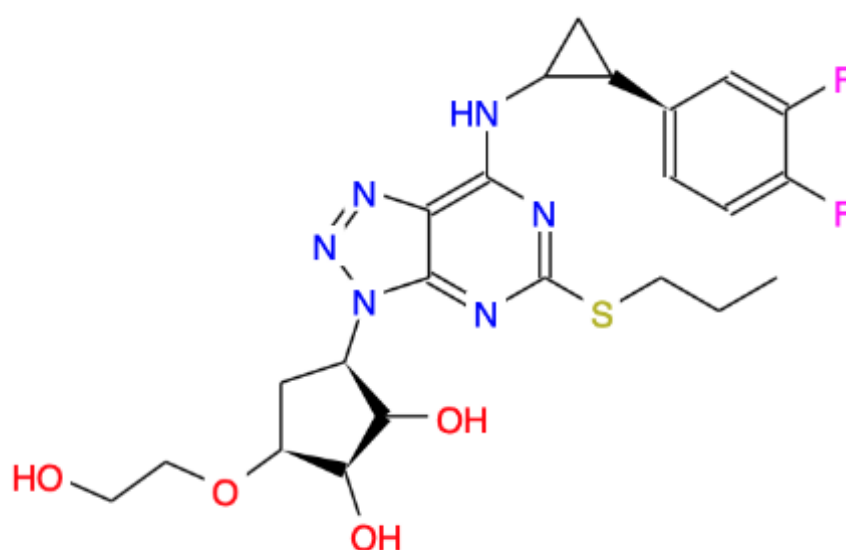
1.2.   Acetylsalicylic acid

ASA, mainly known under the brand name Aspirin®, represents a cornerstone in the pharmacological management of ACS (Yildirim et al., 2017). Besides its analgetic and antiphlogistic effects in higher dosages, aspirin inhibits platelet aggregation by irreversibly blocking the enzyme cyclooxygenase-1, which is responsible for the synthesis of thromboxane A2, a platelet-aggregation-activator, at low doses (Abramson et al., 1985). When taken orally or intravenously ASA is quickly converted into its major metabolite salicylic acid (SA) via hydrolyzation, which performs the main pharmacological actions of ASA (Castillo-Garcia et al., 2015).

Figure 2. Chemical structure of acetylsalicylic acid
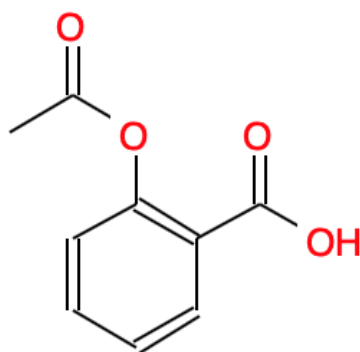
1.3.   Staphylococcus aureus

Staphylococcus aureus (S. aureus) is a clinically relevant gram-positive pathogen. Besides its ability to pose a threat to human health, it is also known to colonize healthy humans without showing any symptoms. However, people who are persistent carriers of *S. aureus* show a higher risk for ensuing infections (Lowy, 1998). *S. aureus* is a leading cause of iatrogenic infections and poses a substantial pressure on the health system (Lister et al., 2014). Above all, its ability to develop resistance against antibiotics, especially methicillin, complicates the treatment of infections due to higher morbidity and mortality rates (Lakhundi et al., 2018). Furthermore, infections attributable to methicillin-resistant *S. aureus* are related to higher costs for hospitals and longer hospital stays in comparison to MSSA infections (Antonanzas et al., 2015). *Staphylococcus aureus* infections include a broad variety of clinical manifestations like skin-, soft tissue-, device-associated-infections, infections of the respiratory tract, osteomyelitis and most importantly it is a major contributor to bacteremia and infective endocarditis (Tong et al., 2015).

1.4.   Coagulase negative staphylococci

Coagulase negative Staphylococci (CoNS) are part of the natural flora of the skin and mucosa, but they can also cause serious infections, typically in health-care settings and especially device-associated infections in vulnerable patients (Becker et al., 2014). Infective endocarditis (IE), attributable to coagulase negative staphylococcal infections, is reported in about 10% of cases. Additionally, the evolution of drug-resistant strains, especially methicillin-resistant and

vancomycin-resistant *S. epidermidis*, complicates the antibiotic management of IE caused by coagulase negative staphylococci (Garcia de la Maria et al., 2015).

1.5. Enterococci

Enterococci belong to the natural intestinal flora of humans, but they are also capable of causing opportunistic infections in susceptible patients. *Enterococcus faecium* (*E. faecium)* and *Enterococcus faecalis* (*E. faecalis)* are most commonly responsible for infections in humans. As pathogens causing nosocomial infections, Enterococci can lead to endocarditis, urinary tract infections, and infections of the central nervous system, among others (Wozniak-Biel et al., 2019). Importantly, *E. faecium* and *E. faecalis* are known to evolve antibiotic resistance, especially to vancomycin (Ayobami et al., 2020).

1.6. Escherichia coli

Escherichia coli (E. coli) is a gram-negative pathogen, that is part of the natural intestinal flora of humans and belongs to the family of *Enterobacteriaceae*. *E. coli* is able to provoke intestinal as well as extraintestinal infections (Croxen et al., 2014) and intestinal infections due to *E. coli* are caused by pathovars (EPEC: enteropathogenic *E. coli*; ETEC: enterotoxigenic *E. coli*; EIEC: enteroinvasive *E. coli*; EAggEC: enteroaggregative *E. coli;* EHEC: enterohemorrhagic *E. coli*) (Kayser et al., 2001).

1.7. Infective endocarditis

Infective endocarditis is defined as an inflammation of the endocardium due to bacteremia caused by different microorganisms entering the bloodstream (Baddour et al., 2015). Patients with artificial heart valves or immunosuppressed patients are more susceptible to developing infective endocarditis; in addition, intravenous drug use, venous catheters, and hemodialysis pose further risks for infective endocarditis (Cahill et al., 2017). Gram-positive bacteria like Staphylococci, Streptococci and Enterococci are mainly associated with IE. *S. aureus*, found in 25 to 30% of cases, is most commonly attributable to this potentially fatal disease*.* Although the incidence of infective endocarditis remains low, the number of cases has escalated in recent years and the increasing emergence of iatrogenic infections with drug resistant bacteria poses a challenge when including the prolonged therapy regimes necessary (Cahill et

al., 2016). IE is a severe disease, with high mortality rates if untreated and even with antibiotic or surgical management, the mortality rate remains at about 18% (Dietz et al., 2012). According to recent guidelines, successful treatment of infective endocarditis is based on eradication of the causative bacterium and in some cases surgical removal of the infected tissue may be necessary. Antibiotic therapy of IE due to staphylococcal infections (*S. aureus* or coagulase-negative staphylococci), typically administered intravenously, depends on whether native heart valves or prosthetic valves are involved and whether methicillin-resistant or methicillin-susceptible staphylococci caused the infection. The standard pharmacological therapy of non-resistant staphylococcal IE involves the use of a beta-lactam, such as oxacillin, cloxacillin or flucloxacillin, taken for 4 - 6 weeks. In patients with prosthetic valves combination-therapy of more than one antibiotic is recommended and for eradication of resistant strains daptomycin, vancomycin, gentamicin, clindamycin are the drugs of choice (Habib et al., 2015).

1.8. Aims

The published data on antimicrobial activity of ticagrelor raised the hypothesis that other cardiovascular drugs might also exhibit unknown antimicrobial activities.

In this study, we performed in-vitro antimicrobial susceptibility testing of ticagrelor and other cardiovascular drugs against a large number of gram-positive and gram-negative bacteria. We focused on pathogens commonly associated with infective endocarditis such as *S. aureus*, coagulase-negative Staphylococci and Enterococci. In order to broaden the test spectrum, we additionally investigated 11 different strains of gram-negative *E. coli*. Furthermore, since antibiotic resistance is an evolving problem all over the world (WHO, 2014), the search for new antibiotic regimes is constantly gaining importance. Following the work of Lancelotti et al. (2019), that demonstrated not only bactericidal properties of ticagrelor against gram-positive bacteria, but also provided evidence of synergistic effects between ticagrelor and some antibiotics (Lancelotti et al., 2019), we explored potential enhancing antimicrobial activities of antiplatelet/antibiotic combinations in an in-vitro synergy screening. Antimicrobial susceptibility testing with ASA and its major metabolite SA was another aim of this study, since DAPT with aspirin and a $P2Y_{12}$ receptor inhibitor is considered a fundamental combination for the treatment of ACS, we investigated on possible synergistic antimicrobial effects of SA and ticagrelor.

2. Materials and methods

2.1. Bacterial strains

Forty bacterial strains, including drug resistant strains, such as methicillin-resistant *Staphylococcus epidermidis* (MRSE), methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant Enterococci, were examined in this study. Thirty-six were routinely collected from positive blood cultures from the Department of Medicine I, Division of Infectious Diseases and Tropical Medicine, Medical University of Vienna, Vienna, Austria. Four bacterial strains were standard reference bacteria: *S. aureus* ATCC 29213 (American Type Culture Collection), *S. aureus* ATCC 33592, *E. coli* ATCC 25922 and *S. aureus* DSMZ 25629 (German Collection of Microorganisms and Cell Cultures). In this study ten different strains of *S. aureus*, ten different strains of coagulase-negative Staphylococci, nine different strains of Enterococci and eleven different strains of *E. coli* were examined (Table 1).

2.2. Isolate preparation

Bacterial strains were stored in freezing vials at -80°C. Bacterial suspensions were transferred from the freezing vials onto Columbia agar plates with 5% sheep blood using an inoculation loop. After overnight incubation, one to two colonies were picked using a sterile cotton swab and were suspended in approximately 4 ml sodium chloride (0.9% NaCl w/v in water) (B. BRAUN MEDICAL). In order to obtain a standardized final inoculum concentration of 10^4 cells per ml on agar plates, a turbidity standard equivalent to 0.5 McFarland was adjusted using a densimeter (DensiCHECK^{TM} plus by BIOMERIEUX, Austria) and this solution was further diluted 1:100 with sodium chloride (0.9% NaCl w/v in water).

2.3. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using two different methods. First, the agar dilution method was used to investigate on antimicrobial susceptibility of seven different cardiovascular drugs, including ticagrelor, as well as two metabolites of clopidogrel and salicylic acid, as major metabolite of aspirin (Table 2). Second, broth-microdilution was performed in order to determine minimum inhibitory concentrations (MICs) of ticagrelor and to investigate on possible combinational antimicrobial activity between ticagrelor and salicylic acid.

Table 1. List of bacterial strains

Strain group	Strain ID	Ampicillin/Oxacillin susceptibility
grampositive		
Staphylococcus aureus	ATCC 29213*	sensitive
Staphylococcus aureus	168/18	resistant
Staphylococcus aureus	DSMZ 25629*	resistant
Staphylococcus aureus	231/20	sensitive
Staphylococcus aureus	249/20	sensitive
Staphylococcus aureus	874/19	resistant
Staphylococcus aureus	280/20	sensitive
Staphylococcus aureus	ATCC 33592*	resistant
Staphylococcus aureus	845/19	resistant
Staphylococcus aureus	204/20	sensitive
Staphylococcus epidermidis	385/13	resistant
Staphylococcus epidermidis	381/13	resistant
Staphylococcus epidermidis	253/13	sensitive
Staphylococcus epidermidis	276/13	sensitive
Staphylococcus epidermidis	410/13	resistant
Staphylococcus epidermidis	255/13	sensitive
Staphylococcus warneri	166/13	sensitive
Staphylococcus warneri	268/13	resistant
Staphylococcus haemolyticus	378/13	sensitive
Staphylococcus haemolyticus	386/13	resistant
Enterococcus faecalis	9/13	sensitive
Enterococcus faecalis	360/13	sensitive
Enterococcus faecalis	356/13	sensitive
Enterococcus faecalis	38/13	sensitive
Enterococcus faecium	278/13	resistant
Enterococcus faecium	280/13	resistant
Enterococcus faecium	219/13	resistant
Enterococcus faecium	193/13	resistant
Enterococcus faecium	212/13	resistant
gramnegative		
Escherichia coli	ATCC 25922*	sensitive
Escherichia coli	372/20	resistant
Escherichia coli	71/20	resistant
Escherichia coli	140/20	resistant
Escherichia coli	391/20	resistant
Escherichia coli	379/20	resistant
Escherichia coli	39/20	sensitive
Escherichia coli	43/20	sensitive
Escherichia coli	49/20	sensitive
Escherichia coli	98/20	resistent
Escherichia coli	262/18	resistent

*standard reference microorganisms; Abbreviations: ATCC: American Type Culture Collection; DSMZ: German Collection of Microorganisms and Cell Cultures GmbH.

2.3.1. Agar dilution

As recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST), we used Mueller-Hinton agar (MHA) plates for antimicrobial susceptibility testing of non-fastidious bacteria (EUCAST, 2020). In the course of agar dilution, two different concentrations of the active substance to be investigated were incorporated into MHA. MHA plates which did not receive the drug were used as positive controls. Ten to eleven different bacterial isolates were applied simultaneously on the surface of one agar plate via 10 µL spots using a pipette (Figure 3). After incubation for 16-20h at 37°C and 40% humidity, susceptibility was defined by visually comparing growth on the drug containing agar plates with that on drug free agar plates. This method was used as a screening for antimicrobial susceptibility as well as for an approximate MIC determination.

Figure 3. Mueller Hinton agar inoculated with *Staphylococcus aureus*
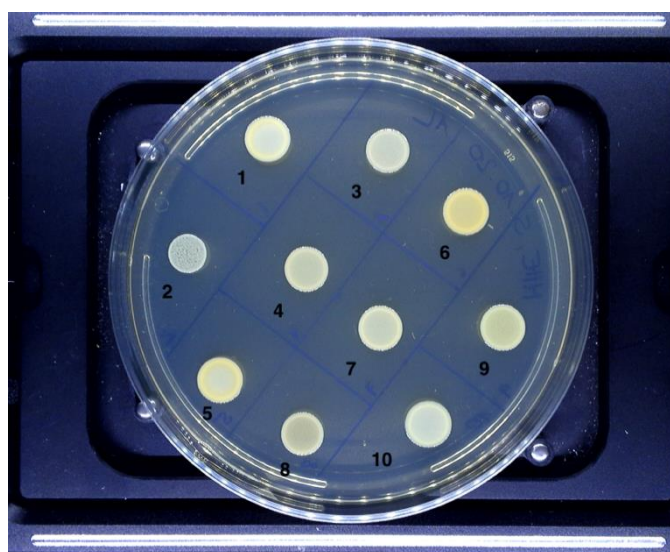
Figure 3 shows 10 µl spots of ten different bacterial strains *of S. aureus* on a MHA plate without any active substance after an incubation period of 16-20h at 37°C and 40% humidity. 1: *S. aureus* ATCC 29213 (S); 2: *S. aureus* 168/18 (R); 3: *S. aureus* DSMZ 25629 (R); 4: *S. aureus* 231/20 (S); 5: *S. aureus* 249/20 (S); 6: *S. aureus* 874/19 (R); 7: *S. aureus* 280/20 (S); 8: *S. aureus* ATCC 33592 (R); 9: *S. aureus* 845/19 (R); 10: *S. aureus* 204/20 (S)

2.3.1.1.   Preparation of agar plates

Mueller-Hinton Agar 2 (Sigma-Aldrich, Austria) was prepared according to the manufacturer's instructions. After autoclaving, the medium was cooled down to approximately 50°C. Depending on the desired final drug concentration, a certain amount of the drug-containing stock-solution was added and this solution was mixed well, using a magnetic stirrer, in order to ensure a steady drug concentration on the whole surface of the agar plate. The plates for the positive control did not receive any drug. The different drug concentrations incorporated into the agar plates were corresponding to a maximum serum concentration (C_{max}) of either 10x C_{max} or 100x C_{max}. (Table 2). Approximately 20 ml of the medium was dispensed into sterile petri dishes under the laminar air flow, using a stripette. After cooling down, the agar plates were stored at 4°C.

2.3.1.2.   Preparation of agar plates with ticagrelor

MHA was prepared as described above. Due to the solubility of ticagrelor (Sigma Aldrich Handels GmbH, Swiss) it was dissolved in dimethyl sulfoxide (DMSO) (PAN^{TM} Biotech, Germany) and considering the toxicity of DMSO, we kept the final concentration of this solvent on our agar plates below 1% to avoid possible antimicrobial effects of DMSO which might have an antibacterial effect on its own. It was very important to keep the temperature of the autoclaved agar solution as well as the drug containing stock solution (100 mg/ml ticagrelor dissolved in DMSO) at 50°C to prevent ticagrelor from precipitating when mixing the stock solution with the agar solution. In order to temperate the agar solution, a magnetic stirrer was used and an Eppendorf Thermomixer (Eppendorf Thermomixer comfort) to temperate the stock solution. By the time, where both solutions were tempered to 50°C, the stock solution was added to the agar solution under the laminar air flow. After mixing, approximately 20 ml of the agar solution was dispensed into sterile petri dishes. After cooling down agar plates were stored at 4°C.

Table 2. mean/median C$_{max}$ values of tested drugs

Drug	Mean/median C$_{max}$* (µg/ml)	Study concentration (mg/L)		Subjects	Dose	References
		~10x C$_{max}$	~100x C$_{max}$			
Ticagrelor	0.81	10	100	Healthy volunteers	Oral administration of 100mg ticagrelor twice/day	(Dobesh et al., 2014)
Acetylsalicylic acid	1.01	10	100	Healthy volunteers	Oral administration of 100mg ASA once/day	(Nagelschmitz et al., 2014)
Salicylic acid**	4.19	50	500	Healthy volunteers	Oral administration of 100mg ASA once/day	(Nagelschmitz et al., 2014)
R-Clopidogrel carboxylic acid***	0.002516	0.03	0.3	Healthy volunteers	Oral administration of 75mg clopidogrel once/day	(Karazniewicz-Lada et al., 2014)
2-oxo-Clopidogrel***	0.0068	0.07	0.7	Healthy volunteers	Oral administration of 75mg clopidogrel once/day	(Li et al., 2015)
Atorvastatin	0.0319	0.5	5	Healthy volunteers	Oral administration of 40mg atorvastatin once/day	(Ghim et al., 2019)
Digitoxin		0.2	2			
Bisoprolol	0.02067	0.25	2.5	Healthy volunteers	Single-dose oral administration of 5mg bisoprolol fumarate	(Tjandrawinata et al., 2012)
Canrenoate	2.066	30	300	Healthy volunteers	Intravenous injection of canrenoate-K 200mg	(Krause et al., 1983)
Valsartan	2.3	-	200	Healthy volunteers	Oral administration of 80mg Valsartan once/day	(Prasad et al., 2002)

*C$_{max}$: maximum plasma concentration; **Salicylic acid as a major metabolite after oral administration of ASA; ***R-Clopidogrel carboxylic acid and 2-oxo-Clopidogrel as major metabolites after oral administration of Clopidogrel. Abbreviations**:** ASA: acetylsalicylic acid; K: potassium.

2.3.2. Broth microdilution with ticagrelor and salicylic acid

Referring to the recommendations of the European Committee on Antimicrobial Susceptibility testing, un-supplemented cation-adjusted Mueller Hinton broth (Merck KgaA, Germany) was used for antimicrobial susceptibility testing with broth microdilution of non-fastidious bacteria (EUCAST, 2020).

The method incorporated a standardized inoculum concentration of 5×10^5 cells per ml and a 16-20h incubation period at 37°C and 40% humidity.

U-bottomed 96-well microtiter plates (Greiner-bio-one, Germany) were utilized. One hundred microliter of the bacterial suspension was dispensed in each well except for the negative control, which contained medium alone. The wells in rows A and D contained 100 µl of a two-fold serial dilution (200 mg/l to 0,39 mg/l) of ticagrelor. The rows B and E contained the same two-fold concentrations of ticagrelor as in the rows A and D plus 50 µl of a stable concentration of salicylic acid (500 mg/l) in order to examine possible combinational effects of ticagrelor and SA. Each well contained a final volume of 200 µl. After incubation at 37°C and 40% humidity, the MIC values were recorded visually as the lowest concentration that inhibits visible bacterial growth.

Figure 4. Microdilution – 96-well microtiter plate
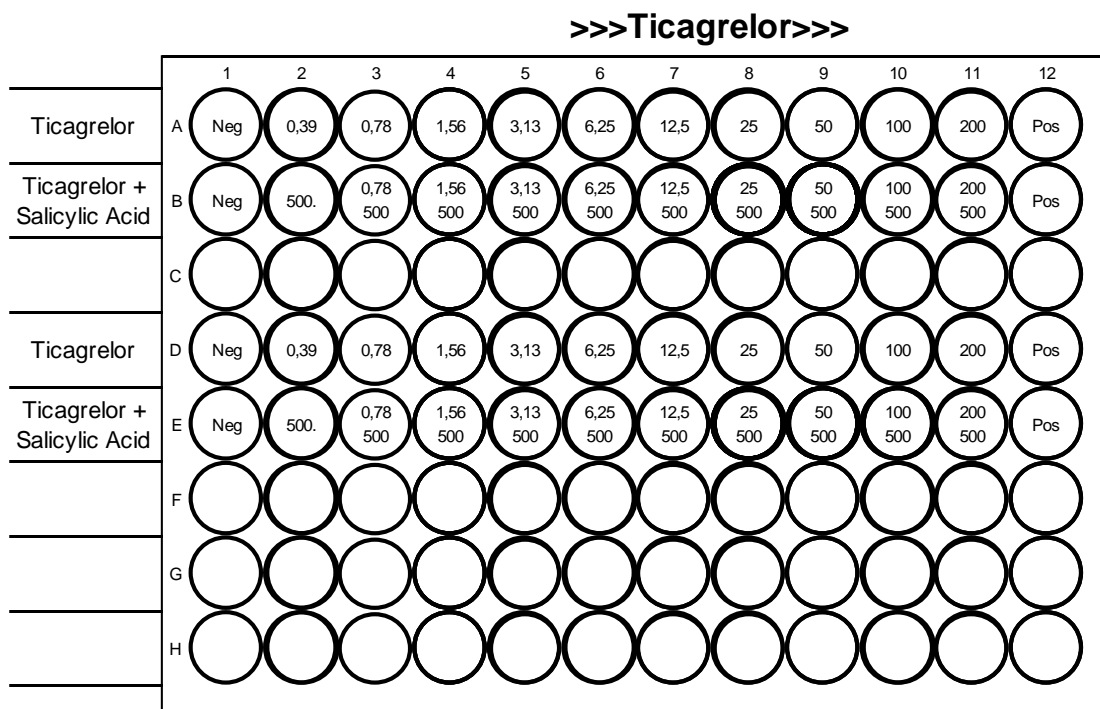
Abbreviations: Pos: positive control; Neg: negative control.

The wells of the microdilution plates A2-A11 and D2-D11 contained serial dilutions of ticagrelor (1:2) starting with a maximum concentration of 200 mg/L in column 11. The wells from B3-B11 contained a constant concentration of salicylic acid (500mg/L) with the same serial dilutions of ticagrelor as above. A volume of 100 µL of a bacterial inoculum equal to a 0.5 McFarland turbidity standard was dispensed into the wells except fort the negative control (column 1). The positive control contained 100 µl of bacterial suspensions plus 100 µl cation adjusted Mueller Hinton broth.

2.3.3. In-vitro synergy-screening of antiplatelet/antibiotic combinations

This screening method incorporated the agar-dilution method, as used previously for antimicrobial susceptibility testing combined with the Epsilometertest (E-test)-method for MIC determination of antibiotics. The E-test method implies a gradually increasing concentration of an antibiotic, integrated into a plastic strip, which is set onto the surface of an agar plate, inoculated with the bacterial strain to be tested. This method is commonly used for MIC determination of antibiotics (bioMerieux, 2020).

E-test strips of cefazolin, dalbavancin, vancomycin, fusidic acid, clindamycin, linezolid, eravacycline, doxycycline, gentamicin and daptomycin were used in this assay (from bioMerieux, Austria and Liofilchem, Austria). This screening method implies MHA plates, incorporated with either ticagrelor or salicylic acid or a combination of salicylic acid and ticagrelor, as well as MH agar plates without any active substance, considered as control plates. MHA plates were supplemented with either 10 mg/l ticagrelor, 500 mg/l SA or 10 mg/l ticagrelor combined with 500 mg/l SA (triple-combination). The agar plates were prepared as described previously in Chapter 2.3.1.1..

Six *S. aureus* isolates, that showed susceptibility to ticagrelor in previous antimicrobial susceptibility testing, were examined. Three of them were resistant against methicillin and three were sensible to methicillin. One MSSA and one MRSA strain was a standard laboratory strain.

S. aureus isolates:

- ATCC 33592 (R)
- 845/19 (R)
- 168/18 (R)
- ATCC 29213 (S)
- 249/20 (S)
- 280/20 (S)

Bacterial strains were isolated as described previously in Chapter 2.2.. The bacterial suspensions of each isolate, adjusted to a turbidity standard of 0.5 McFarland in 2 ml sodium chloride (0.9% NaCl w/v in water), were applied onto the surface of the agar plates using sterile cotton swabs. After that, the E-test strips were placed onto the surface of the agar plates using sterile forceps and those plates were incubated for 16-24h at 37°C and 40% humidity. After the incubation period, the MIC values were taken visually, according to the manufacturer's recommendations (bioMerieux, Austria / Liofilchem, Austria).

Figure 5. Mueller Hinton agar plate without any active substance, inoculated with *Staphylococcus aureus* 845/19 (R) and an Epsilometer test-strip of linezolid

Figure 5 shows a MH agar plate without any active substance (control plate) after an incubation period of 16-20h at 37°C and 40% humidity. A bacterial suspension adjusted to a turbidity standard of 0.5 McFarland of *S. aureus* 845/19 was applied via cotton swab and an E-test strip of linezolid (bioMerieux, Austria) was placed onto the surface.

3. Results

3.1. Minimum inhibitory concentrations by agar dilution

Tables 3-6 show the MIC values of 40 bacterial strains with the corresponding drugs or metabolites. Ticagrelor inhibited visible bacterial growth of 28 gram-positive bacteria at concentrations up to 100 mg/l: nine strains of coagulase negative Staphylococci, nine strains of Enterococci and ten strains of *S. aureus*. Ticagrelor was ineffective against all gram-negative strains of *E. coli*.

S. haemolyticus 386/13 showed resistance against ticagrelor at concentrations up to 100 mg/l.

S. warneri 166/13 was susceptible to canrenoate at concentrations up to 300 mg/l. Although salicylic acid did not inhibit bacterial growth of any gram-positive bacterial strain at concentrations of 500 mg/l, we observed a reduced intensity of bacterial growth compared to the control plate.

Figure 6. Mueller Hinton agar plate without active substance, inoculated with ten strains of coagulase negative staphylococci
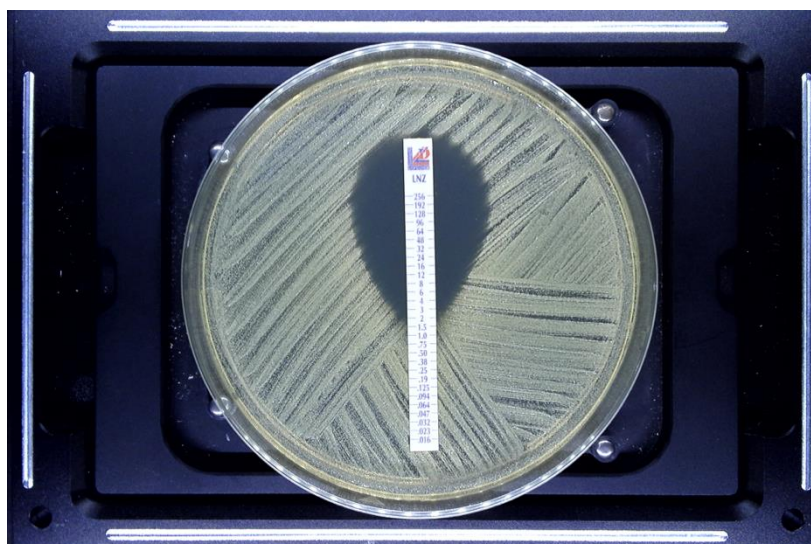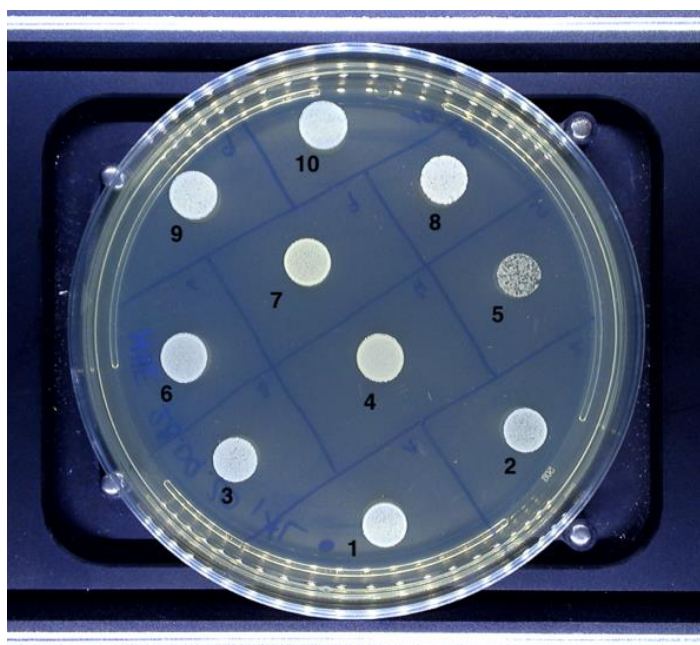

Figure 6 shows a MHA plate without any added drug (control plate) after an incubation period of 16-20h at 37°C and 40% humidity. 10 µl spots of bacterial suspensions of ten different strains of coagulase negative Staphylococci were inoculated simultaneously onto one agar plate. 1: S. epidermidis 385/13; 2: S. epidermidis 381/13 (R); 3: S. epidermidis 253/13 (S); 4: S. epidermidis

276/13 (S); 5: S. epidermidis 410/13 (R); 6: S. epidermidis 255/13 (S); 7: S. warneri 268/13 (S); 8:
S. warneri 166/13 (S); 9: S. haemolyticus 378/13 (S); 10: S. haemolyticus 386/13 (R)

Figure 7. Mueller Hinton agar plate with ticagrelor, inoculated with ten different strains of
coagulase negative staphylococci
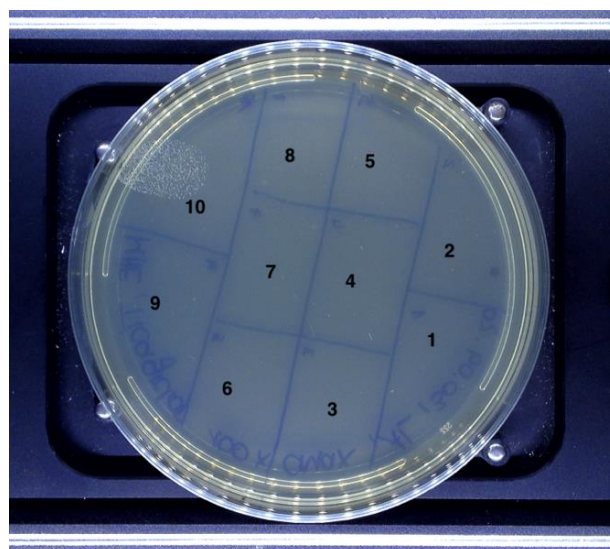
Figure 7 shows a MHA plate incorporated with ticagrelor (100 mg/l) after an incubation period
of 16-20h at 37°C and 40% humidity. The agar plates in figure 6 and figure 7 were
simultaneously inoculated with the same bacterial suspensions of ten different strains of
coagulase negative Staphylococci. The strain identifications are reported in the table below.
At concentrations of 100 mg/l ticagrelor, all bacterial strains except *Staphylococcus
haemolyticus 386/13* (Number 10) were inhibited. *S. haemolyticus* 386/13 appears to be
resistant against ticagrelor at concentrations up to 100 mg/l. 1: S. epidermidis 385/13; 2: S.
epidermidis 381/13 (R); 3: S. epidermidis 253/13 (S); 4: S. epidermidis 276/13 (S); 5: S.
epidermidis 410/13 (R); 6: S. epidermidis 255/13 (S); 7: S. warneri 268/13 (S); 8: S. warneri
166/13 (S); 9: S. haemolyticus 378/13 (S); 10: S. haemolyticus 386/13 (R)

Table 3. Minimum inhibitory concentrations by agar dilution *(Staphylococcus aureus)*

Compound	Minimum inhibitory concentration (MIC) in mg/l									
	Strains tested									
	S. aureus ATCC* 29213 (S)	S. aureus 168/18 (R)	S. aureus DSMZ* 25629 (R)	S. aureus 231/20 (S)	S. aureus 249/20 (S)	S. aureus 874/19 (R)	S. aureus 280/20 (S)	S. aureus ATCC* 33592 (R)	S. aureus 845/19 (R)	S. aureus 204/20 (S)
-Ticagrelor	100	100	100	100	100	100	100	100	100	100
- R-Clopidogrel carboxylic acid**	>0.3	>0.3	>0.3	>0.3	>0.3	>0.3	>0.3	>0.3	>0.3	>0.3
- 2-oxo-Clopidogrel**	>0.7	>0.7	>0.7	>0.7	>0.7	>0.7	>0.7	>0.7	>0.7	>0.7
- Acetylsalicylic acid	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
- Salicylic acid***	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
- Atorvastatin	>5	>5	>5	>5	>5	>5	>5	>5	>5	>5
- Digitoxin	>2	>2	>2	>2	>2	>2	>2	>2	>2	>2
- Canrenoate	>300	>300	>300	>300	>300	>300	>300	>300	>300	>300
- Bisoprolol	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5
- Valsartan	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200

* standard reference microorganisms; **R-Clopidogrel Carboxylic acid and 2-oxo Clopidogrel as major metabolites after oral administration of Clopidogrel; ***salicylic acid as major metabolite after oral administration of Acetylsalicylic acid; Abbreviations: (S): Oxacillin/Ampicillin sensible; (R): Oxacillin/Ampicillin resistant; ATCC: American Type Culture Collection; DSMZ: German Collection of Microorganisms and Cell Cultures GmbH.

Table 4. Minimum inhibitory concentrations by agar dilution (coagulase negative Staphylococci)

Compound	Minimum inhibitory concentration (MIC) in mg/l									
	Strains tested									
	S. epidermidis 385/13 (R)	S. epidermidis 381/13 (R)	S. epidermidis 253/13 (S)	S. epidermidis 276/13 (S)	S. epidermidis 410/13 (R)	S. epidermidis 255/13 (S)	S. warneri 166/13 (S)	S. warneri 268/13 (R)	S. haemolyticus 378/13 (S)	S. haemolyticus 386/13 (R)
- Ticagrelor	100	100	100	100	100	100	100	100	100	>100
- R-Clopidogrel carboxylic acid*	>0.3	>0.3	>0.3	>0.3	>0.3	>0.3	>0.3	>0.3	>0.3	>0.3
- 2-oxo-	>0.7	>0.7	>0.7	>0.7	>0.7	>0.7	>0.7	>0.7	>0.7	>0.7
- Acetylsalicylic	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
- Salicylic acid**	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
- Atorvastatin	>5	>5	>5	>5	>5	>5	>5	>5	>5	>5
- Digitoxin	>2	>2	>2	>2	>2	>2	>2	>2	>2	>2
- Canrenoate	>300	>300	>300	>300	>300	>300	300	>300	>300	>300
- Bisoprolol	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5
- Valsartan	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200

*R-Clopidogrel Carboxylic acid and 2-oxo Clopidogrel as major metabolites after oral administration of Clopidogrel; **salicylic acid as major metabolite after oral administration of Acetylsalicylic acid; Abbreviations: (S): Oxacillin/Ampicillin sensible; (R): Oxacillin/Ampicillin resistant.

Table 5. Minimum inhibitory concentrations by agar dilution (Enterococci)

Compound	Minimum inhibitory concentration (MIC) in mg/l								
	Strains tested								
	E. faecalis 9/13 (S)	*E. faecalis* 360/13 (S)	*E. faecalis* 356/13 (S)	*E. faecalis* 38/13 (S)	*E. faecium* 278/13 (R)	*E. faecium* 280/13 (R)	*E. faecium* 219/13 (R)	*E. faecium* 193/13 (R)	*E. faecium* 212/13 (R)
- **Ticagrelor**	100	100	100	100	100	100	100	100	100
- **R-Clopidogrel carboxylic acid***	>0.3	>0.3	>0.3	>0.3	>0.3	>0.3	>0.3	>0.3	>0.3
- **2-oxo-Clopidogrel***	>0.7	>0.7	>0.7	>0.7	>0.7	>0.7	>0.7	>0.7	>0.7
- **Acetylsalicylic acid**	>100	>100	>100	>100	>100	>100	>100	>100	>100
- **Salicylic acid***	>500	>500	>500	>500	>500	>500	>500	>500	>500
- **Atorvastatin**	>5	>5	>5	>5	>5	>5	>5	>5	>5
- **Digitoxin**	>2	>2	>2	>2	>2	>2	>2	>2	>2
- **Canrenoate**	>300	>300	>300	>300	>300	>300	>300	>300	>300
- **Bisoprolol**	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5
- **Valsartan**	>200	>200	>200	>200	>200	>200	>200	>200	>200

*R-Clopidogrel Carboxylic acid and 2-oxo Clopidogrel as major metabolites after oral administration of Clopidogrel; **salicylic acid as major metabolite after oral administration of acetylsalicylic acid; Abbreviations: (S): Oxacillin/Ampicillin sensible; (R): Oxacillin/Ampicillin resistant.

Table 6. Minimum inhibitory concentrations by agar dilution (*Escherichia coli*)

Compound	Minimum inhibitory concentration (MIC) in mg/l										
	Strains tested										
	E. coli 372/20 (R)	*E. coli* 71/20 (R)	*E. coli* 140/20 (R)	*E. coli* 391/20 (R)	*E. coli* 379/20 (R)	*E. coli* 39/20 (S)	*E. coli* 43/20 (S)	*E. coli* 49/20 (S)	*E. coli* 98/20 (R)	*E. coli* ATCC* 25922 (S)	*E. coli* 262/18 (R)
- Ticagrelor	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
- R-Clopidogrel carboxylic acid**	>0.3	>0.3	>0.3	>0.3	>0.3	>0.3	>0.3	>0.3	>0.3	>0.3	>0.3
- 2-oxo-Clopidogrel**	>0.7	>0.7	>0.7	>0.7	>0.7	>0.7	>0.7	>0.7	>0.7	>0.7	>0.7
- Acetylsalicylic acid	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
- Salicylic acid***	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
- Atorvastatin	>5	>5	>5	>5	>5	>5	>5	>5	>5	>5	>5
- Digitoxin	>2	>2	>2	>2	>2	>2	>2	>2	>2	>2	>2
- Canrenoate	>300	>300	>300	>300	>300	>300	>300	>300	>300	>300	>300
- Bisoprolol	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5	>300	>2.5
- Valsartan	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200

* standard reference microorganisms; **R-Clopidogrel Carboxylic acid and 2-oxo Clopidogrel as major metabolites after oral administration of Clopidogrel; ***salicylic acid as major metabolite after oral administration of Acetylsalicylic acid; Abbreviations: (S): Oxacillin/Ampicillin sensible; (R): Oxacillin/Ampicillin resistant; ATCC: American Type Culture Collection.

3.2. Minimum inhibitory concentrations (MICs) of ticagrelor by broth microdilution and synergy testing

The aim of broth microdilution was on the one hand to determine definite MIC values of ticagrelor, since agar dilution was used for antimicrobial susceptibility screening, and on the other hand to investigate possible enhancing effects regarding antimicrobial activity between ticagrelor and salicylic acid.

Tables 7 - 10 show the MIC values observed in the broth microdilution assay. The MICs of ticagrelor against Enterococci ranged from 12,5 mg/l – 100 mg/l. Whereas, no antimicrobial activity could be observed in gram-negative *E. coli*, which correlates with the results of agar dilution. MIC values of ticagrelor against ten strains of *S. aureus* ranged from 25 mg/l – 100 mg/l. MICs of ticagrelor against coagulase negative Staphylococci ranged from 25 mg/l – 50 mg/l. Resistance of *Staphylococcus haemolyticus* 386/13 against ticagrelor was confirmed with this method.

Referring to the agar dilution method, the concentrations of ticagrelor, which were incorporated into the agar, were 10 mg/l and 100 mg/l. Since 28 bacterial strains showed MICs underneath 100 mg/l, except for 11 strains of *E. coli* and *S. haemolyticus* 386/13, these findings are in close agreement with the agar dilution method.

Enhancing effects between ticagrelor and SA were observed with *Staphylococcus epidermidis 253/13, Staphylococcus epidermidis 410/13, Staphylococcus epidermidis 255/13, Staphylococcus warneri 166/13, Staphylococcus haemolyticus 378/13, Staphylococcus aureus 168/18, Enterococcus faecalis 9/13, Enterococcus faecalis 356/13, Enterococcus faecalis 360/13* and *Enterococcus faecalis 38/13.*

Figure 8. Microtiter plate inoculated with bacterial suspensions after incubation

Figure 8 shows visible bacterial growth of *Staphylococcus epidermidis 410/13* in the wells A2-A7, D2-D7, B2, E2, A12, B12, C12, D12, E12. Combinational antimicrobial effects between ticagrelor and SA could be observed (see pipetting scheme in Figure 4). The combination was able to reduce the MIC value of ticagrelor against *S. epidermidis* 410/13 from 25 mg/l (ticagrelor only) to 0.78125 mg/l (ticagrelor + 500 mg/l SA)

Table 7. Minimum inhibitory concentration of coagulase negative Staphylococci by broth microdilution

Bacterial strain	MIC ticagrelor	MIC ticagrelor + SA
S. epidermidis 385/13 (R)	50mg/l	50mg/l + 500mg/l
S. epidermidis 381/13 (R)	25mg/l	25mg/l + 500mg/l
S. epidermidis 253/13 (S)	25mg/l	0,78125mg/l + 500mg/l
S. epidermidis 276/13 (S)	25mg/l	25mg/l + 500mg/l
S. epidermidis 410/13 (R)	25mg/l	0,78125mg/l + 500mg/l
S. epidermidis 255/13 (S)	25mg/l	0,78125mg/l + 500mg/l
S. warneri 268/13 (R)	25mg/l	25mg/l + 500mg/l
S. warneri 166/13 (S)	25mg/l	0,78125mg/l + 500mg/l
S. haemolyticus 378/13 (S)	25mg/l	0,78125mg/l + 500mg/l
S. haemolyticus 386/13 (R)	>200mg/l	>200mg/l + 500mg/l

Table 7 shows the MIC values of ticagrelor and the MIC values of ticagrelor in combination with 500mg/l salicylic acid against 10 strains of coagulase negative Staphylococci obtained by broth-microdilution. Abbreviations: MIC: Minimum inhibitory concentration; SA: salicylic acid; (R): Oxacillin/Ampicillin resistant; (S): Oxacillin/Ampicillin sensible.

Table 8. Minimum inhibitory concentration of *Staphylococcus aureus* by broth microdilution

Bacterial strain	MIC ticagrelor	MIC Ticagrelor + SA
S. aureus ATCC 29213 (S)	100mg/l	100mg/l + 500mg/l
S. aureus 168/18 (R)	25mg/l	0,78125mg/l + 500mg/l
S. aureus DSMZ 25629 (R)	100mg/l	100mg/l + 500mg/l
S. aureus 231/20 (S)	100mg/l	100mg/l + 500mg/l
S. aureus 249/20 (S)	50mg/l	50mg/l + 500mg/l
S. aureus 874/19 (R)	100mg/l	100mg/l + 500mg/l
S. aureus 280/20 (S)	100mg/l	100mg/l + 500mg/l
S. aureus ATCC 33592 (R)	50mg/l	50mg/l +500 mg/l
S. aureus 845/19 (R)	50mg/l	50mg/l + 500 mg/l
S. aureus 204/20 (S)	100mg/l	100mg/l + 500mg/l

Table 8 shows the MIC values of ticagrelor and the MIC values of ticagrelor in combination with 500mg/l salicylic acid against 10 strains *Staphylococcus aureus* obtained by broth-microdilution. Abbreviations: ATCC: American Type Culture Collection; DSMZ: German Collection of Microorganisms and Cell Cultures GmbH; MIC: Minimum inhibitory concentration; SA: salicylic acid; (R): Oxacillin/Ampicillin resistant; (S): Oxacillin/Ampicillin sensible.

Table 9. Minimum inhibitory concentration of Enterococci by broth microdilution

Bacterial strain	MIC ticagrelor	MIC Ticagrelor + SA
E. faecalis 9/13 (S)	100mg/l	6,25mg/l + 500mg/l
E. faecium 212/13 (R)	100mg/l	100mg/l + 500mg/l
E. faecium 278/13 (R)	-	-
E. faecalis 356/13 (S)	12,5mg/l	6,25mg/l + 500mg/l
E. faecalis 360/13 (S)	25mg/l	12,5mg/l + 500mg/l
E. faecalis 38/13 (S)	12,5mg/l	3,125 mg/l + 500mg/l
E. faecium 193/13 (R)	50mg/l	50mg/l + 500mg/l
E. faecium 219/13 (R)	-	-
E. faecium 280/13 (R)	-	-

Table 9 shows the MIC values of ticagrelor and the MIC values of ticagrelor in combination with 500mg/l salicylic acid against 6 strains of Enterococci obtained by broth-microdilution. Abbreviations: MIC: Minimum inhibitory concentration; SA: salicylic acid; (R): Oxacillin/Ampicillin resistant; (S): Oxacillin/Ampicillin sensible.

Table 10. Minimum inhibitory concentration of *Escherichia coli* by broth microdilution

Bacterial strain	MIC ticagrelor	MIC Ticagrelor + SA
E. coli 372/20 (R)	> 200mg/l	> 200mg/l + >500mg/l
E. coli 71/20 (R)	> 200mg/l	> 200mg/l + >500mg/l
E. coli 140/20 (R)	> 200mg/l	> 200mg/l + >500mg/l
E. coli 391/20 (R)	> 200mg/l	> 200mg/l + >500mg/l
E. coli 379/20 (R)	> 200mg/l	> 200mg/l + >500mg/l
E. coli 39/20 (S)	> 200mg/l	> 200mg/l + >500mg/l
E. coli 43/20 (S)	> 200mg/l	> 200mg/l + >500mg/l
E. coli 49/20 (S)	> 200mg/l	> 200mg/l + >500mg/l
E. coli 98/20 (R)	> 200mg/l	> 200mg/l + >500mg/l
E. coli 262/18 (R)	> 200mg/l	> 200mg/l + >500mg/l
E. coli ATCC 22592 (S)	> 200mg/l	> 200mg/l + >500mg/l

Table 10 shows the MIC values of ticagrelor and the MIC values of ticagrelor in combination with 500mg/l salicylic acid against 11 strains of *Escherichia coli* obtained by broth-microdilution. Abbreviations: ATCC: American Type Culture Collection; MIC: Minimum inhibitory concentration; SA: salicylic acid; (R): Oxacillin/Ampicillin resistant; (S): Oxacillin/Ampicillin sensible.

3.3.   Antiplatelet/antimicrobial synergy screening

This screening method was used to investigate on the potential additive properties, regarding antimicrobial activity, of combinations of ticagrelor with different antibiotics and salicylic acid with antibiotics.

The concentrations of ticagrelor and SA were chosen based on the results of agar dilution as the highest static concentration that allows a screening for all pathogens, because inhibition of growth has not yet occurred.

The MIC values of the E-test strips were read where the edge of the inhibition ellipse crosses the scale of the strip. Tables 11-12 show the MIC values of each antibiotic against 6 different S*. aureus* isolates on 4 different agar plates. A total of 232 combinations of antibiotics, active substance and bacteria were tested.

The changes of MIC values of the tested antibiotics by E-tests due to the addition of ticagrelor, salicylic acid, and the combination of salicylic acid with ticagrelor are presented in the figures 9-14 as the percentage difference in MICs compared to un-supplemented MHA (control plate).
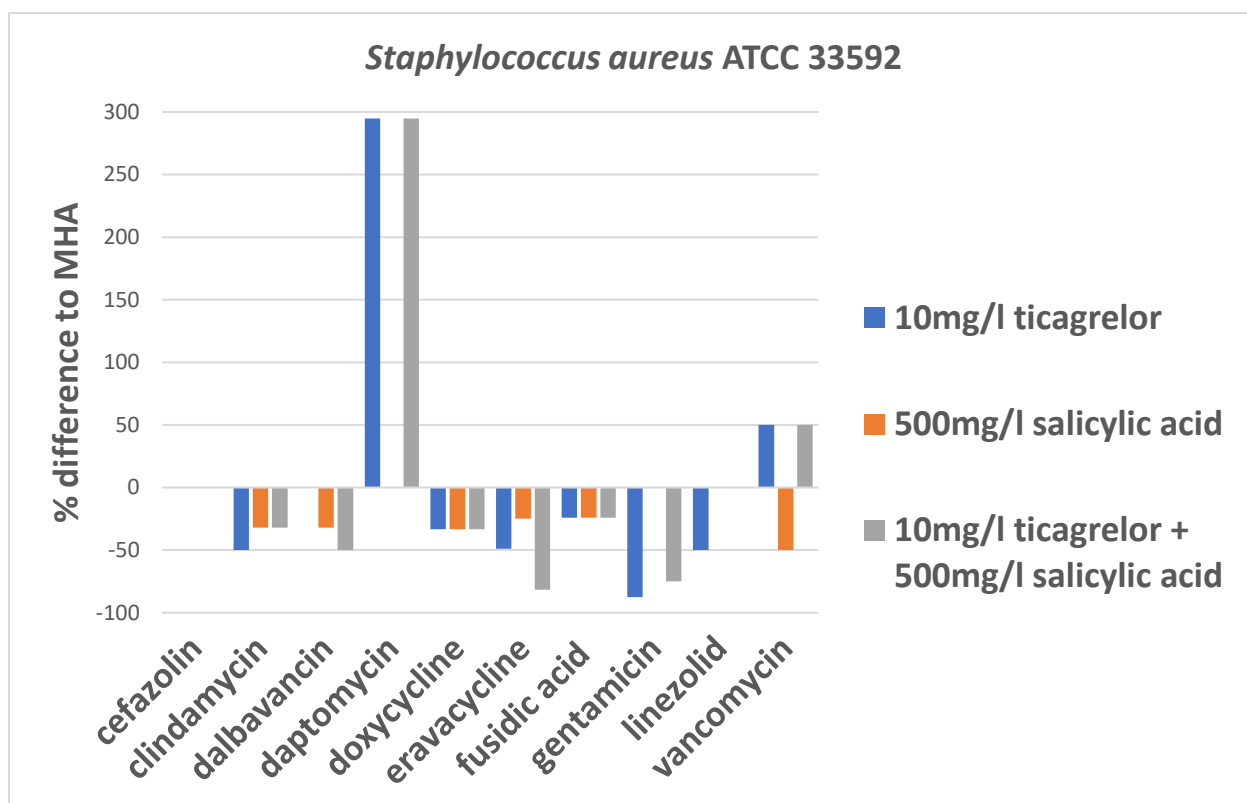
Figure 9. Synergy-screening wit *Staphylococcus aureus* ATCC 33592 (R): Changes of the MICs of antibiotics against *S. aureus* ATCC 33592 by E-test due to the addition of ticagrelor, salicylic acid, salicylic acid and ticagrelor, compared to E-tests on un-supplemented Mueller Hinton agar.
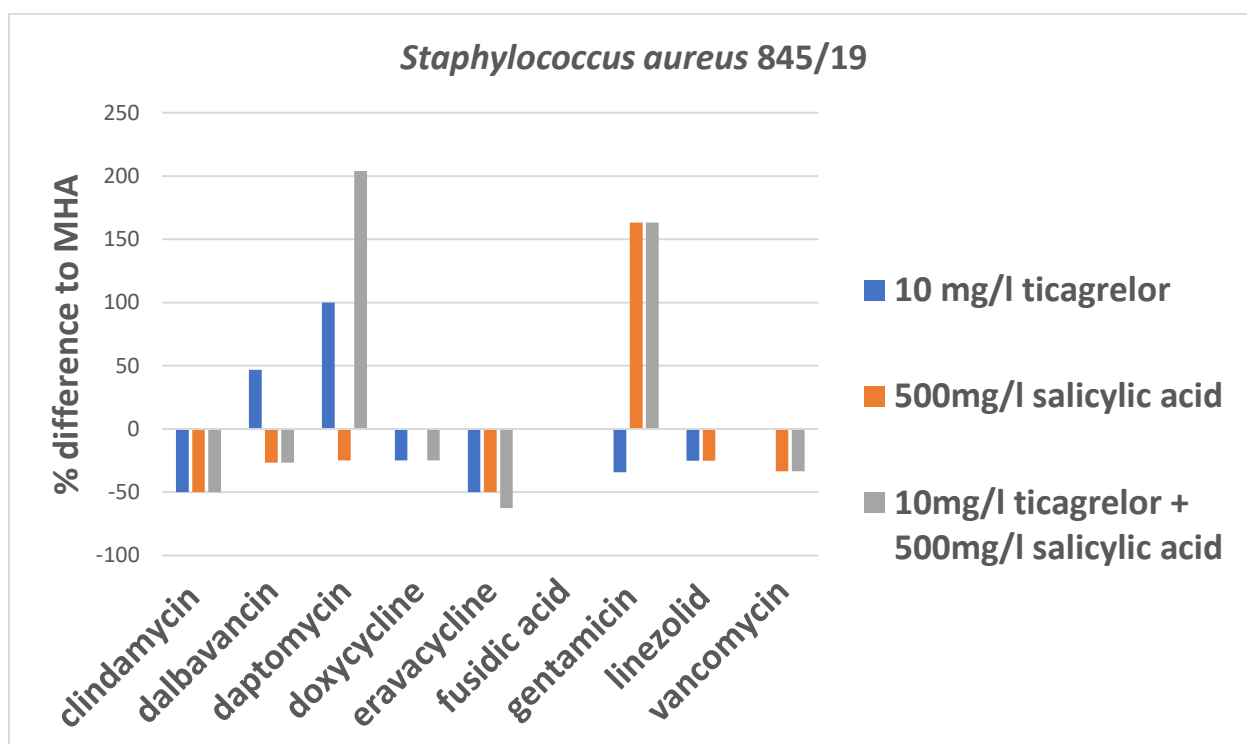
Figure 10. Synergy-screening with *Staphylococcus aureus* 845/19 (R): Changes of the MICs of antibiotics against *S. aureus* 845/19 by E-test due to the addition of ticagrelor, salicylic acid, salicylic acid and ticagrelor, compared to E-tests on un-supplemented Mueller Hinton agar.

Figure 11. Synergy-screening with *Staphylococcus aureus* 168/18 (R): Changes of the MICs of antibiotics against *S. aureus* 168/18 by E-test due to the addition of ticagrelor, salicylic acid, salicylic acid and ticagrelor, compared to E-tests on un-supplemented Mueller Hinton agar.
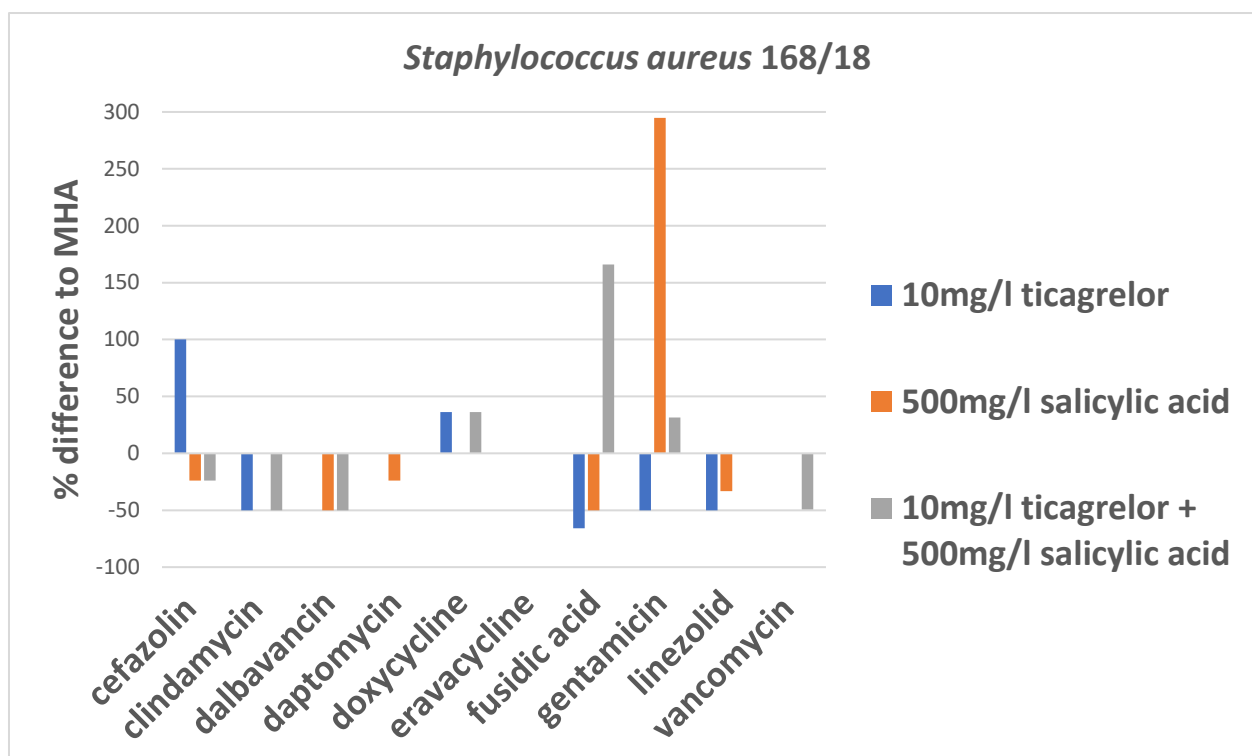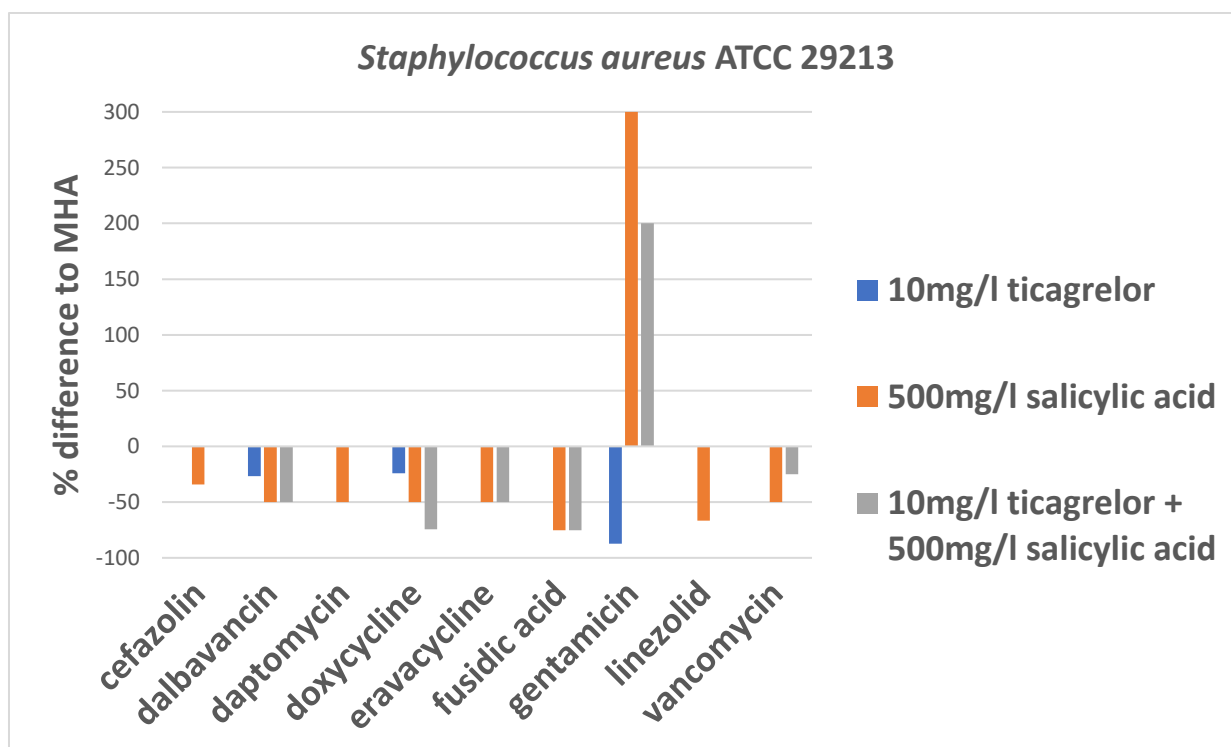

Figure 12. Synergy-screening with *Staphylococcus aureus* ATCC 29213 (S): Changes of the MICs of antibiotics against *S. aureus* ATCC 29213 by E-test due to the addition of ticagrelor, salicylic acid, salicylic acid and ticagrelor, compared to E-tests on un-supplemented Mueller Hinton agar.

Figure 13. Synergy-screening with *Staphylococcus aureus* 249/20 (S): Changes of MICs of the antibiotics against *S. aureus* 249/20 by E-test due to the addition of ticagrelor, salicylic acid, salicylic acid and ticagrelor, compared to E-tests on un-supplemented Mueller Hinton agar.

Figure 14. Synergy-screening with *Staphylococcus aureus* 280/20 (S): Changes of the MICs of antibiotics against *S. aureus* 280/20 by E-test due to the addition of ticagrelor, salicylic acid, salicylic acid and ticagrelor, compared to E-tests on un-supplemented Mueller Hinton agar.
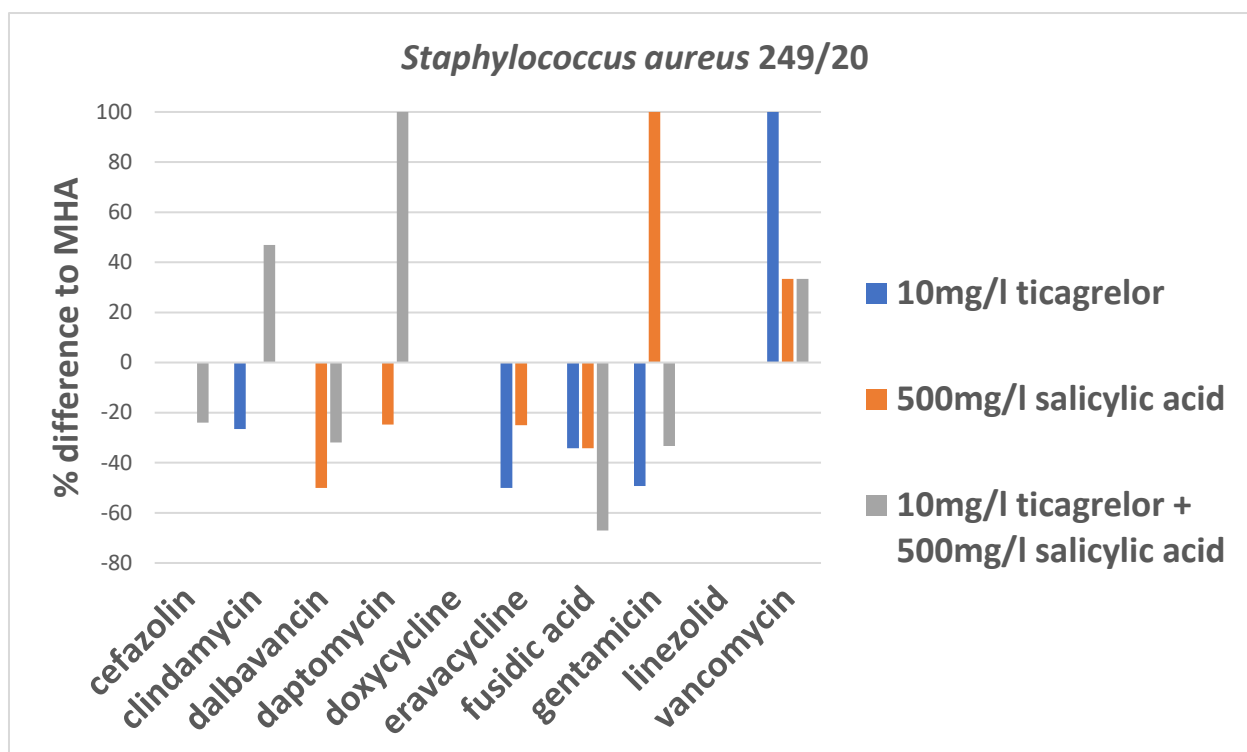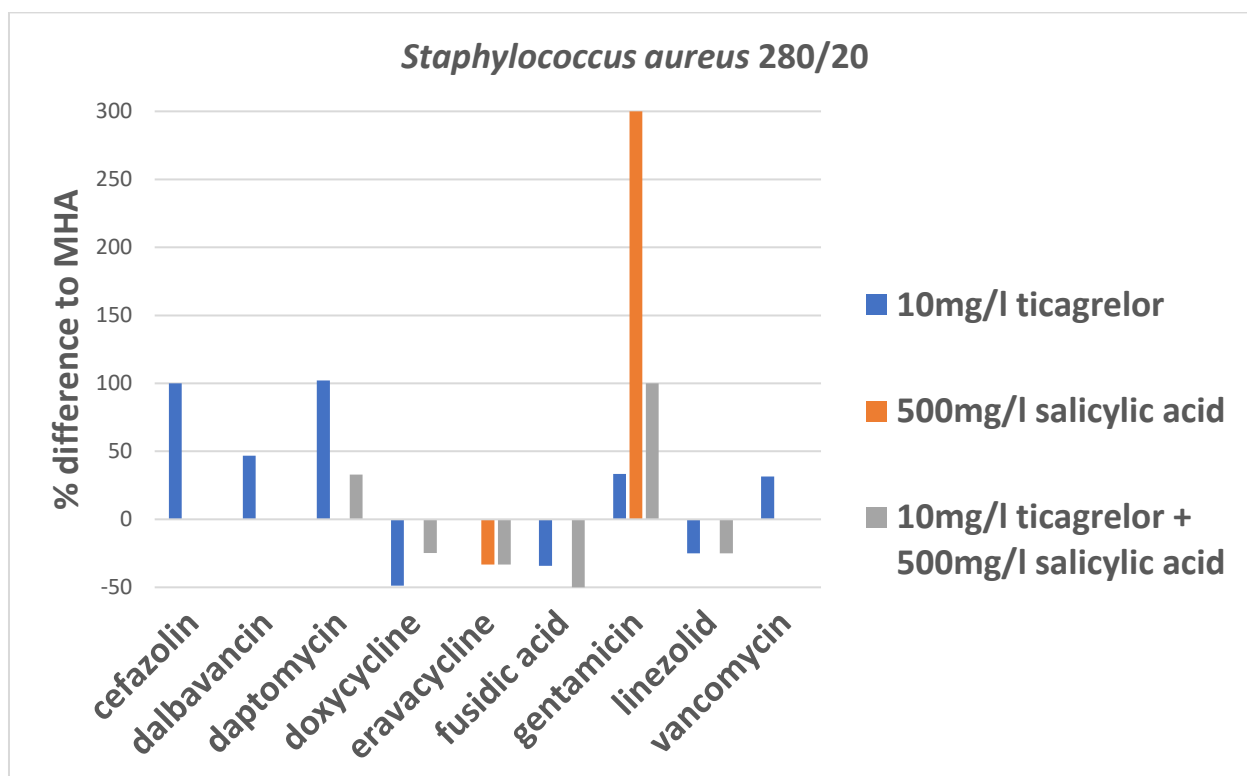
3.3.1. Changes in Minimum Inhibitory Concentrations

The increases and decreases of MICs reported in the following paragraph describe only changes of MICs at 50% or higher.

S. aureus ATCC 33592 (R):

Ticagrelor decreased the MIC of linezolid (-50,0%), clindamycin (-50,0%), gentamicin (-87,5%). SA decreased the MIC of vancomycin (-50,0%). The combination of salicylic acid and ticagrelor decreased the MIC of gentamicin (-75%), eravacyline (-81,6%) and dalbavancin (-50,0%). Ticagrelor increased the MIC of daptomycin (+294,7%) and the MIC of vancomycin (+50,0%). The combination of ticagrelor and SA increased the MIC of daptomycin (+294,74%).

S. aureus 845/19 (R):

Ticagrelor decreased the MIC of clindamycin (-50,0%) and eravacyline (-50%). SA decreased the MIC of eravacycline (-50%) and clindamycin (-50,0%). SA in combination with ticagrelor decreased the MIC of eravacycline (-62,5%) and clindamycin (-50,0%). Ticagrelor increased the MIC of daptomycin (+100,0%). SA increased the MIC of gentamicin (+163,2%). The combination of SA and ticagrelor increased the MIC gentamicin (+163,2%) and daptomycin (+204,0%).

S. aureus 168/18 (R):

Ticagrelor decreased the MIC of clindamycin (-50,0%), fusidic acid (-67,0%), gentamicin (-50,0%) and linezolid (-50,0%). SA decreased the MIC of dalbavancin (-50,0%) and fusidic acid (-50,0%). The combination of ticagrelor an salicylic acid decreased the MIC of dalbavancin (-50,0%), clindamycin (-50,0%). Ticagrelor increased the MIC cefazolin (+100,0%). SA increased the MIC of gentamicin (+294,7%). The combination of SA and ticagrelor increased the MIC of fusidic acid (+167,0%).

S. aureus ATCC 29213 (S):

Ticagrelor decreased the MIC of gentamicin (-87,2%). Salicylic acid decreased the MIC of vancomycin (-50,0%), linezolid (-66,7%), fusidic acid (-75,3%), eravacycline (-50,0%), doxycycline (-50,0%), daptomycin (-50,0%) and dalbavancin (-50,0%).

The combination of ticagrelor and salicylic acid decreased the MIC of fusidic acid (-75,3%), eravacycline (-50,0%), doxycycline (-74,4%) and dalbavancin (-50,0%). Salicylic acid increased the MIC of gentamicin (+300,0%). The combination of salicylic acid and ticagrelor increased the MIC of gentamicin (+200,0%).

S. aureus 249/20 (S):

Ticagrelor decreased the MIC of eravacycline (-50,0%). SA decreased the MIC of dalbavancin (-50,0%). The combination of ticagrelor and SA decreased the MIC of fusidic acid (-67,1%). Ticagrelor increased the MIC of vancomycin (+100,0%). SA increased the MIC of gentamicin (+100,0%). The combination of SA and ticagrelor increased the MIC of daptomycin (+100,0%).

S. aureus 280/20 (S):

The combination of SA and ticagrelor decreased the MIC of fusidic acid (-50,0%). Ticagrelor increased the MIC of cefazolin (+100,0%) and daptomycin (+102,1%). Salicylic acid increased the MIC of gentamicin (+300,0%). The combination of ticagrelor and salicylic acid increased the MIC of gentamicin (+100,0%).

Figure 15. Synergy-screening with gentamicin (Epsilometertest) and ticagrelor against ATCC 33592 on Mueller-Hinton agar plates

Plate 1. Control plate

Plate 2. 10 mg/l ticagrelor

MIC: 0.75 µg/ml
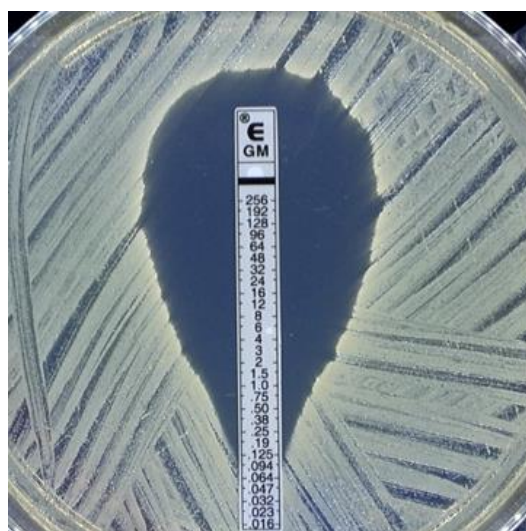
MIC: 0.125 µg/ml

Plate 3. 500 mg/l salicylic acid   Plate 4. 500 mg/l SA + 10 mg/l ticagrelor
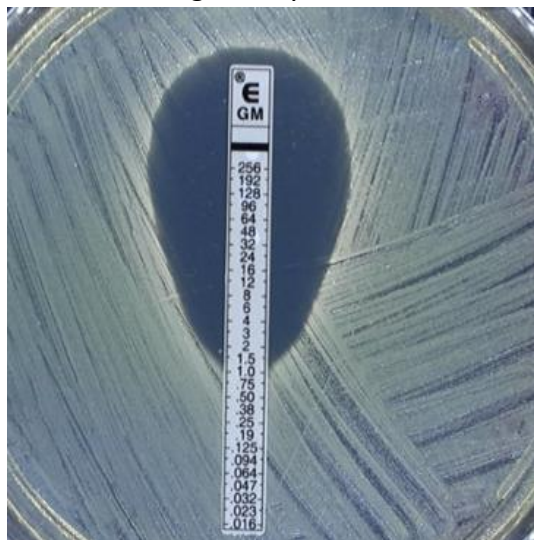
MIC: 1,5 µg/ml   MIC: 0.25 µg/ml

The pictures show 4 different Mueller Hinton agar plates inoculated with a bacterial suspension of *S. aureus* ATCC 33592 (turbidity standard: 0,5 McFarland) and an E-test strip of gentamicin on the surface of the agar plates, after an incubation period of 16-20h at 37°C and 40% humidity. Plate 1 does not contain any active substance. Plate 2 contains 10 mg/l Ticagrelor. Plate 3 contains 500 mg/l salicylic acid and plate 4 contains a combination of 500 mg/l salicylic acid with 10 mg/l ticagrelor.

Ticagrelor reduced the MIC of gentamicin from 0.75 µg/ml (Plate 1) to 0.125 µg/ml (Plate 2). Salicylic acid increased the MIC from 0.75 µg/ml (Plate 1) to 1.5 µg/ml (Plate 3). Plate 4 shows that the combination of SA and ticagrelor reduced the MIC from 0.75µg/ml to 0.25 µg/ml.

Table 11. Minimum inhibitory concentration by Epsilometertest (methicillin-resistant *Staphylococcus aureus*)

antimicrobial agent	*S. aureus* ATCC 33592				*S. aureus* 845/19				*S. aureus* 168/18			
	MHA*	10mg/l Ticagrelor	500mg/l SA	Ticagrelor + SA	control	10mg/l Ticagrelor	500mg/l SA	Ticagrelor +SA	control	10mg/l Ticagrelor	500mg/l SA	Ticagrelor +SA
					Epsilometertest**							
Cefazolin	128	128	128	128	12	>256	96	>256	0,5	1	0,38	0,38
Clindamycin	0,094	0,047	0,064	0,064	0,094	0,047	0,047	0,047	0,064	0,032	0,064	0,032
Dalbavancin	0,094	0,094	0,064	0,047	0,064	0,094	0,047	0,047	0,094	0,094	0,047	0,047
Daptomycin	0,19	0,75	0,19	0,75	0,125	0,25	0,094	0,38	0,25	0,25	0,19	NA***
Doxycycline	12	8	8	8	0,125	0,094	0,125	0,094	0,047	0,064	0,047	0,064
Eravacyline	0,125	0,064	0,094	0,023	0,016	0,008	0,008	0,006	0,008	0,008	0,008	0,008
Fusidic acid	0,25	0,19	0,19	0,19	0,25	0,25	0,25	0,25	0,094	0,032	0,047	0,25
Gentamicin	1	0,125	1	0,25	0,19	0,125	0,5	0,5	0,38	0,19	1,5	0,5
Linezolid	3	1,5	3	3	2	1,5	1,5	2	1,5	0,75	1	1,5
Vancomycin	1	1,5	0,5	1,5	0,75	0,75	0,5	0,5	0,75	0,75	0,75	0,38

*control plate without any active substance; ** concentration of the antimicrobial substance in µg/ml; ***no answer; Abbreviations: SA: salicylic acid; Abbreviations: ATCC: American Type Culture Collection; MHA: Mueller Hinton agar.

Table 12. Minimum inhibitory concentration by Epsilometertest (methicillin-sensible *Staphylococcus aureus*)

antimicrobial agent	S. aureus ATCC 29213				S. aureus 249/20				S. aureus 280/20			
	MHA*	10mg/l Ticagrelor	500mg/l SA	Ticagrelor +SA	control	10mg/l Ticagrelor	500mg/l SA	Ticagrelor +SA	control	10mg/l Ticagrelor	500mg/l SA	Ticagrelor +SA
	Epsilometertest**											
Cefazolin	0,38	0,38	0,25	0,38	0,25	0,25	0,25	0,19	0,25	0,5	0,25	0,25
Clindamycin	NA***	NA	NA	NA	0,064	0,047	0,064	0,094	NA	NA	NA	NA
Dalbavancin	0,064	0,047	0,032	0,032	0,094	0,094	0,047	0,064	0,064	0,094	0,064	0.047
Daptomycin	0,25	0,25	0,125	0,25	0,125	0,125	0,094	0,25	0,094	0,19	0,094	0,125
Doxycycline	0,25	0,19	0,125	0,064	0,19	0,19	0,19	0,19	0,125	0,064	0,125	0,094
Eravacycline	0,016	0,016	0,008	0,008	0,016	0,008	0,012	0,016	0,012	0,012	0,008	0,008
Fusidic acid	0,38	0,38	0,094	0,094	0,38	0,25	0,25	0,125	0,38	0,25	0,38	0,19
Gentamicin	0,5	0,064	2	1,5	0,75	0,38	1,5	0,5	3	4	12	6
Linezolid	3	3	1	3	2	2	2	2	4	3	4	3
Vancomycin	1	1	0,5	0,75	0,75	1,5	1	1	0,38	0,5	0,38	0,38

*control plate without any active substance; **concentration of the antimicrobial substance in μg/ml; ***no answer; Abbreviations: SA: salicylic acid; Abbreviations: ATCC: American Type Culture Collection; MHA: Mueller Hinton agar.

4. Discussion

4.1. In-vitro antimicrobial activity of ticagrelor

The emergence of methicillin-resistant *S. aureus* strains is constantly rising, and the severity of these infections combined with increased mortality rates are more than challenging. In addition, the rising use of intravascular catheters and prosthetic devices leads to an increased risk population (Bamberger et al., 2007).

Furthermore, the growing onset of hospital-acquired infections due to drug-resistant *E. faecium* complicates pharmacological treatment strategies. The therapy of infective endocarditis caused by resistant strains of *E. faecium* poses a problem due to the lack of bactericidal therapeutic options (Munita et al., 2012).

As mentioned in the introduction, there has been increasing evidence on antimicrobial properties of ticagrelor. Lancelotti et al. (2019) demonstrated bactericidal properties of ticagrelor in time-kill assays against ten different bacterial strains, including one MRSA, one MRSE, one MSSA and one VRE. However, some limitations are noted in this work such as the low number of bacterial isolates tested. To overcome these disadvantages, 29 gram-positive bacteria and eleven gram-negative bacteria were subjected to antimicrobial susceptibility testing in the present work. The large number of bacterial isolates that were investigated, is a key strength of the present study.

Supporting and expanding the findings of Lancelotti et al. (2019), antibacterial activity of ticagrelor was found against 28 different gram-positive bacteria, inclusively drug-resistant strains like MRSE, MRSA and methicillin-resistant Enterococci.

Whereas we found *S. haemolyticus 386/13*, belonging to the family of coagulase negative staphylococci, to be resistant against ticagrelor at concentrations up to 100 mg/l.

Antimicrobial susceptibility was investigated using the agar dilution-method and definite MIC values of ticagrelor were evaluated using the microdilution method. Since agar dilution was rather used as a screening-method, only two widely differing concentrations (12,3x c_{max} = 10 mg/l vs. 123x c_{max} = 100 mg/l) were examined in this course. After screening, the broth microdilution method was used to precisely determine MIC values of ticagrelor. Although MICs evaluated by agar dilution compared to broth microdilution differ, agreement can be reported in those results. MIC values generated by broth microdilution were at 100 mg/l or lower, but higher than 10 mg/l, except for one resistant gram-positive strain (*S. haemolyticus*

386/13), that showed resistance in both methods. The investigation of antimicrobial activity of ticagrelor against 11 gram-negative bacteria like *E. coli* proved ineffective at concentrations up to 100mg/l. These results correlate with the findings of Lancelotti et al. (2019), who describe ineffectiveness against gram-negative bacterial strains at concentrations up to 80 mg/l.

The lowest concentration of ticagrelor, that prevented visible bacterial growth was 12,5 mg/l against *E. faecalis* 356/13 (S) and *E. faecalis* 38/13 (S) and the concentration of 25 mg/l inhibited growth of two MRSE isolates (*S. epidermidis* 385/13 and *S. epidermidis* 381/13), whereas Lancelotti et al. (2019) describe the MIC against one MRSE strain as 30 mg/l.

Ticagrelor proved effective against all five MRSA strains, as well as against all 5 MSSA strains and the lowest concentration of ticagrelor inhibiting one MRSA strain (*S. aureus* 168/18) was found to be 25 mg/l, followed by 50mg/l against *S. aureus* ATCC 33592 (R) and *S. aureus* 845/19 (R). However, some limitations arouse with these results, because the concentrations of ticagrelor that inhibited bacterial growth in this in-vitro experiment are more than ten times higher than systemically reached values of C_{max} after conventional dosages like 100 mg twice a day orally (C_{max} = 0.81 mg/l) (Dobesh et al., 2014). Nevertheless, it was interesting that the microdilution method showed, that the addition of 500 mg/l salicylic acid to ticagrelor decreased the MIC values of ticagrelor significantly against *S. epidermidis* 253/13, *S. epidermidis* 410/13, *S. epidermidis* 255/13, S*. warneri* 166/13, S. haemolyticus 378/13, *S. aureus* 168/18 from 25 mg/l ticagrelor alone to 0,78125 mg/l ticagrelor. A concentration of 0,78125mg/l could be reached using C_{max} values after normal antiplatelet dosages, which is very promising. Enhancing effects with a minor reduction of the MIC values could also be observed in four strains of Enterococci. Although the concentrations of SA are beyond physiological concentrations, these findings are interesting, because the combination of salicylic acid, as a major metabolite of ASA (Castillo-Garcia et al., 2015) and ticagrelor represents a very common drug combination. In the course of DAPT patients usually receive a $P2Y_{12}$ receptor inhibitor concomitantly with aspirin for at least one year (Collet et al., 2020, Ibanez et al., 2017). Referring to the long duration of dual antiplatelet therapy after myocardial infarction, a combinational antibacterial side effect to this therapy is of clinical importance. Therefore, especially for patients at risk for gram-positive infections, ticagrelor should be the drug of choice. Taking into consideration, that only the concentration of 500 mg/l was examined in the course of microdilution, the concentrations of salicylic acid, required

for amplifying antimicrobial activity and for reducing the MIC of ticagrelor to physiologically achieved levels, might be lower than 500 mg/l. In order to make sure, that not SA alone is responsible for those antimicrobial effects, one well containing only 500 mg/l SA was used as control in our microdilution assay. Therefore, we can be sure that this effect occurs only due to the combination of ticagrelor and salicylic acid. To clarify the definite concentrations required for a synergistic effect between SA and ticagrelor, subsequent studies using the Chequerboard method should be performed in the future.

The findings of researchers establishing superiority of DAPT consisting of ticagrelor and ASA compared to clopidogrel and ASA, in patients after a STEMI, suffering from gram-positive infections (Rigatelli et al., 2019) support our findings and demonstrate, that the concentrations in humans, needed for an antimicrobial effect, may be lower compared to in-vitro results. In addition, the enhancing antimicrobial effects of SA and ticagrelor, that we reported, might also explain the lower concentrations in humans, that were necessary for this effect. Furthermore, previously published data provided additional evidence on anti-inflammatory effects of ticagrelor due to modified levels of inflammatory markers like interleukins (Jiang et al., 2018, Sexton et al., 2018). This might also be an explanation to the beneficial effects of ticagrelor in patients with DAPT consisting of ticagrelor and ASA, suffering from gram-positive infections. Additionally, this fact could be another possible reason why much higher concentrations, than physiologically achieved, are necessary in in-vitro experiments. In spite of the limitations due to the in-vitro concentrations of ticagrelor and salicylic acid, these findings warrant future in-vivo investigations regarding required bactericidal concentrations.

4.2.   In-vitro antimicrobial activity of other cardiovascular drugs

The published data about antimicrobial properties of ticagrelor raised the hypothesis that other $P2Y_{12}$ inhibitors, like clopidogrel and also other cardiovascular drugs may have antimicrobial properties too. Therefore, we performed antimicrobial susceptibility testing with two major metabolites of clopidogrel, but it showed ineffective against all 40 bacterial strains at tested concentrations.

Additionally, this work provides data regarding in-vitro antimicrobial susceptibility of atorvastatin, digitoxin, canrenoate, bisoprolol and valsartan. There was no antimicrobial

activity of these substances, at tested concentrations, observed, except for canrenoate against *S. warneri* 166/13 (S).

The fact that clopidogrel metabolites do not exhibit antibacterial activity suggests that this effect does not occur through the inhibition of the $P2Y_{12}$ receptor. Moreover, Lancelotti et al. (2019) did not detect in vitro antimicrobial properties of the active metabolite of prasugrel, another agent from the group of $P2y_{12}$ inhibitors. Suggesting, that the antimicrobial effect of ticagrelor is a unique property of this substance and therefore should be further examined.

4.3. Antimicrobial/antiplatelet drug combinations

Since its development, the therapy of bacterial infections with antibiotics has been accompanied by the continuous emergence of antibiotic resistance. A circumstance that makes therapy increasingly difficult. Multidrug-resistant bacteria such as MRSA and VRE are particularly problematic, due to their ability to cause life-threatening infections, like infective endocarditis, among others (Karaman et al., 2020).

In a recent study, researchers established some combinational bactericidal effects of ticagrelor and antibiotics like vancomycin, rifampicin and ciprofloxacin against resistant gram-positive cocci in a disk diffusion assay (Jean et al., 2019). Inspired by these findings and the findings of Lancelotti et al. (2019), who reported combinational effects between some antibiotics and ticagrelor, we examined how the MICs of the tested antibiotics change due to the addition of ticagrelor, salicylic acid, or the combination of ticagrelor and SA. For this purpose, we performed a synergy screening that combined the agar-dilution method with the E-test method. Considering *S. aureus* as most commonly attributable to infective endocarditis and bacteremia we focused this synergy screening on three MSSA and three MRSA isolates. Based on the magnitude of MIC change, this work provides suggestions of additive, antagonistic, or indifferent behavior of three MRSA and three MSSA isolates tested in 232 combinations of antibiotics, bacteria, ticagrelor and salicylic acid.

Additive behavior was most frequently reported due to the combination of ticagrelor and clindamycin, ticagrelor and gentamicin, ticagrelor and eravacycline. Ticagrelor reduced the MIC of clindamycin by 50% against all MRSA isolates tested.

The combination of gentamicin and ticagrelor showed a reduction of the MIC of at least 50% against two MSSA and two MRSA isolates. Referring to recent guidelines, clindamycin in combination with cotrimoxazole can be considered as one possible option for the

pharmacological treatment of native valve endocarditis due to MRSA infections, whereas gentamicin is part of the treatment strategy against native valve endocarditis due to MSSA or MRSA (Habib et al., 2015). In patients with existing DAPT, consisting of ASA and ticagrelor, diagnosed with infective endocarditis, clindamycin and gentamicin might be more potent considering antimicrobial activity.

Surprisingly, this screening assay also showed some unexpected antagonistic effects. Antagonistic behavior was most frequently shown by the combination of salicylic acid and gentamicin (in 5 of 6 tested *S. aureus* strains). In some cases, the MIC of gentamicin was increased by 300% due to this combination. However, the concentrations of SA that led to this increase in the MIC of gentamicin are 100 times higher than the concentrations reached by this metabolite after an intake of 100 mg ASA per day (Nagelschmitz et al., 2014). Ticagrelor was also able to show some antagonistic effects. Most importantly, in two out of three MRSA isolates, ticagrelor increased the MIC of daptomycin significantly to 100% in *S. aureus* 845/19 and to almost 300% in *S. aureus* ATCC 33592, which is somewhat disappointing, since daptomycin plays an important role in the pharmacological management of endocarditis due to MRSA infections (Habib et al., 2015). However, these results can only be indications and therefore, the exact concentrations for these opposite effects should be further investigated using the chequerboard method.

In summary, ticagrelor was able to reduce the MIC of some antibiotics significantly, but we also reported some increases of the MICs of antibiotics due to the addition of ticagrelor. In this assay, a stable concentration of 10 mg/l was incorporated into the agar for all six *S. aureus* isolates. Therefore, we do not know if possibly lower ticagrelor concentrations show combinational or antagonistic effects with the tested antibiotics. Although these are only in-vitro screening results, it can be assumed that ticagrelor is also thought to have an anti-inflammatory effect via alteration of pro-inflammatory cytokines, which cannot be examined in in-vitro experiments, hence in-vivo investigations could be even more promising.

The method we used for this screening is not recommended for definite synergy testing. Nevertheless, this screening assay gave us the possibility to quickly, easily and cheaply test a large number of drug/bacteria/antibiotic combinations. Although limitations arouse to this method, the provided indications for amplifying and opposite antimicrobial effects encourage future investigations using the chequerboard method in order to determine definitive

synergies or antagonisms and to evaluate necessary concentrations of ticagrelor and salicylic acid for these effects. Definitive synergy results could guide the therapy of infections with MRSA or MSSA, in patients with existing DAPT, consisting of ticagrelor and ASA, and facilitate the decision with which antibiotic to treat.

5. Conclusion

The purpose of the current work was to perform different in-vitro test systems to investigate on antimicrobial properties of ticagrelor and other cardiovascular drugs with a large number of different gram-positive and gram-negative bacterial species.

This investigation has demonstrated bactericidal activity of ticagrelor against 28 gram-positive bacterial strains. Additionally, this work provides indications for synergistic activity between SA, the major metabolite of ASA, and ticagrelor. Although the in-vitro concentrations required for antibacterial effects were beyond systemically reached levels, these findings are useful for expanding the knowledge of antimicrobial activities of ticagrelor, taken concomitantly with ASA in the course of DAPT.

Another aim of this work was to perform an in-vitro synergy-screening to investigate on amplifying antimicrobial effects of antiplatelet / antibiotic combinations. This investigation has shown some significant MIC reductions of antibiotics due to the addition of ticagrelor and/or SA, but some unexpected increases in MIC values were detected as well. Continued efforts are needed to determine exact synergies between antibiotics and ticagrelor as well as to clarify the concentrations of SA required for synergistic activity in combination with ticagrelor.

Notwithstanding the limitations to this work, the study provides additional evidence on antimicrobial activity of ticagrelor and indicates for enhancing effects between ticagrelor and antibiotics as well as between SA and ticagrelor.

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