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Antibiotic drug development

Carina Sofia Silva Matias

PhD Thesis

December 2019

The Novo Nordisk Foundation Center for Biosustainability

Technical University of Denmark

PREFACE

The work described in this thesis was carried from the 1st of January 2017 to the 31st of December 2019.

The work was mainly developed between UNION Therapeutics A/S and The Novo Nordisk Foundation Center for Biosustainability at the Technical University of Denmark (DTU).

It included a two-month research stay at Birmingham's University in the Institute of Microbiology and Infection (IMI) at Professor Ian Henderson group and, a three-week research stay at Utrecht University Medical Center at the Medical Microbiology department in the group of Professor Rob Willems.

The thesis was supervised by Professor Morten Otto Alexander Sommer affiliated with The Novo Nordisk Foundation Center for Biosustainability at DTU and, co-supervised by Professor Hanne Ingmer, affiliated to the University of Copenhagen. The work was funded by the European Union through the "Train2Target" Marie Curie Fellowship (grant agreement No 721484).

Carina Sofia Silva Matias, 31st December 2019

Novo Nordisk Foundation Center for Biosustainability, DTU, Kgs. Lyngby

ABSTRACT

The extensive use of antibiotics, in both clinical and agricultural settings, are one of the major factors contributing to the emergence of antibiotic resistance. Such resistance, in combination with the lack of development of novel antibiotics heightens the urgency to develop novel and effective therapies for the treatment of infectious diseases.

This thesis describes efforts to contribute to the field of antibiotic drug discovery, by focusing on repurposing niclosamide (here by named ATx201) as a potent decolonization agent targeting *Staphylococcus aureus* in atopic dermatitis lesions. In addition, by using *S. aureus* as the model organism, the novel mode of action of ATx201 towards bacteria is described, together with a reduced likelihood of developing resistance and ability to reduce *S. aureus* bacterial load in atopic dermatitis lesions.

Given the consistent antimicrobial properties of ATx201 towards Gram-positive bacteria, specifically *S. aureus,* we further investigated the antimicrobial potential and potential improvement of other pharmacological properties of newly synthesized ATx201 derivatives against a broader panel of clinically important bacteria, including multidrug resistant Gram-negative bacteria. Here, several derivatives were identified as active and we showed that when in combination with a compound that disrupts the outer membrane, ATx201 and other derivatives are potentiated against Gram-negative bacteria, specifically *Escherichia coli*. Overall, our data suggests that ATx201 and derivatives could represent a potent alternative for the treatment of infections caused by multidrug resistant bacteria.

Additionally, we evaluated the resistance rates of *S. aureus* (recovered from environmental settings) towards antibiotics used in clinical and veterinary practice. Given the high rates of resistance observed, this study further highlights the need to develop new antibiotics.

In conclusion, the work conducted in this thesis contributes to the field of antibiotic drug discovery by providing knowledge about possible new antibiotic therapies, with low potential to generate resistance in bacteria.

DANSK RESUMÉ

Den omfattende anvendelse af antibiotika i både klinisk og veterinær praksis er en af de vigtigste årsager til stigningen i antibiotikaresistens. Den øgede resistens kombineret med manglende udbud af nye antibiotika understreger behovet for en prioriteret indsats inden for udvikling af nye og effektive behandlinger af infektionssygdomme.

Denne afhandling omhandler forskning inden for antibiotikaudvikling og fokuserer på en ny anvendelse af niclosamid (ATx201), der har potentiale som et potent antibiotikum rettet mod kolonisering af *Staphylococcus aureus* hos patienter med atopisk dermatitis. Med *S. aureus* som modelorganisme beskriver vi her ATx201s nye virkningsmåde og dets egenskaber; lav risiko for resistensudvikling og bevaring af mikrobiomdiversiteten i huden.

Med udgangspunkt i ATx201s antimikrobielle egenskaber mod Gram-positive bakterier, specifikt *S. aureus*, undersøgte vi det antimikrobielle potentiale og øvrige potentiale for forbedring af andre farmakologiske egenskaber i nyligt syntetiserede ATx201-derivater imod et bredere panel af klinisk vigtige bakterier, herunder multiresistente Gram-negative bakterier. Her blev flere derivater identificeret som aktive stoffer, og vi viste, at i kombination med andre stoffer som destabiliserer bakteriernes ydre membran, blev flere af derivaternes virkning potentieret mod Gram-negative bakterier, specifikt *Escherichia coli*. Samlet set tyder vores resultater på, at ATx201 og dets derivater kunne repræsentere et potent alternativ til behandling af infektioner forårsaget af multiresistente bakterier.

Derudover evaluerede vi resistensudviklingen for *S. aureus* over for antibiotika anvendt i klinisk og veterinær praksis. I betragtning af den høje grad af resistens fremhæver denne undersøgelse yderligere behovet for at udvikle nye antibiotika.

Afslutningsvis bidrager resultaterne fra denne afhandling til ny viden om mulige nye antibiotika-baserede behandlinger med en lav risiko for bakteriel resistensudvikling.

OUTLINE OF THE PHD THESIS

The PhD thesis is divided in five chapters. In chapter one a general introduction to the work conducted in the thesis is provided. Chapters two to four are presented in the form of research papers covering the experimental results of the thesis. The last chapter consists of a short summary of main findings and future perspectives.

Chapter I consists of an introductory chapter, describing the antibiotic resistance crisis and the need to develop novel antimicrobial therapies. It gives an overview about the antibiotic drug development stages, as well alternative approaches for the development of novel antibiotics, with special focus on repurposing existing drugs, such niclosamide.

Chapter II focuses on Manuscript I, which presents a study were ATx201 (niclosamide) was identified as potent antimicrobial agent towards Gram-positive bacteria, in specific *Staphylococcus aureus.* We describe ATx201 as a potential treatment for decolonizing *Staphylococcus aureus* in atopic dermatitis lesions, from early stage preclinical up to stage II clinical trials.

Chapter III includes Manuscript II that describes UNION Therapeutics A/S efforts to develop ATx201 derivative compounds, with focus on improving compound's antimicrobial potency and pharmacological properties. These data will not be published outside the thesis and due to IP interests structures related to derivative compounds will not be included in the document.

Chapter IV presents Manuscript III regarding the genomic and phenotypic characterization of antibiotic resistance in veterinary settings. The study enhances the consequences of antibiotic resistance and the need to develop new antimicrobial treatments.

Chapter V consists of a summary and perspectives of the work conducted during the PhD.

LIST OF MANUSCRIPTS

Overview of scientific manuscripts included in this thesis.

Manuscript 1:

ATx201 reduces *Staphylococcus aureus* colonization and increases Shannon diversity of the skin microbiome in atopic dermatitis patients.

Anne Weiss*, Emilie Delavenne*, Carina Matias*, Heimo Lagler*, Daniel Simon, Ping Li, Jon U. Hansen, Teresa Pires dos Santos, Bimal Jana, Petra Priemel, Christine Bangert, Martin Bauer, Sabine Eberl, Alina Nussbaumer-Pröll, Zoe Anne Österreicher, Peter Matzneller, Tamara Quint, Maria Weber, Hanne Mørck Nielsen, Thomas Rades, Helle Krogh Johansen, Henrik Westh, Wooseong Kim, Eleftherios Mylonakis, Christian Friis, Luca Guardarabassi, John Pace, Carina Lundberg, Rasmus Toft-Kehler, Emma Guttman-Yassky, Georg Stingl, Markus Zeitlinger, Morten Sommer. (*manuscript under revision at Science Translational Medicine, February 2020*)

Manuscript 2:

Evaluation of the *in vitro* activity of ATx201 and derivative compounds against multidrug-resistant bacteria.

Carina Matias, Anne Weiss, Hanne Ingmer, Morten O.A. Sommer.

Manuscript 3:

Genomic epidemiology and characterization of antimicrobial resistance profile of *Staphylococcus aureus* isolates associated with bovine subclinical mastitis.

Carina Matias *, Simone S. Li*, Yasser S. Mahmmod, Line Svennesen, Hanne Ingmer, Morten O.A. Sommer. (*manuscript in preparation*)

* denotes equal contribution.

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Moving to Denmark to pursue my PhD was one of the best decisions I ever made. It allowed me, not only to study and focus on a topic that I found extremely interesting and motivating, but also to challenge myself and to grow, both at professional and personal levels.
Throughout my PhD I also had the opportunity to make acquaintance and meet amazing people, that I want to acknowledge.

Firstly, **Morten**, I'm grateful for the opportunity not only to join UNION Therapeutics but also to be part of your group at DTU. Thank you for all the great discussions and confidence in me and my work. To **Rasmus**, thank you as well for the opportunity to join UNION and for all the encouraging words. To **Gitte**, the "mom" of the Sommer lab, a huge thank you for all the help with the administrative stuff, all the nice conversations and always being present when needed. To all other UNION past and current members, **Christian**, **Wanja**, **Sune**, **Mads**, **Morten B.**, **Morten J.**, **Pascal** and **Anne W**. thank you for the nice get-togethers' and Christmas lunches! Also, to **Anne W.** thank you for being so helpful with the *H. pylori* experiments and always being available when I needed help around the lab. Finally, thank you **Sune** for the abstract translation.

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CHAPTER I - INTRODUCTION

ANTIBIOTIC RESISTANCE

Infectious diseases were always a challenge to human health. Before antibiotics were discovered, diseases caused by microorganisms, such as bacteria, represented one of the leading causes of mortality worldwide [1,2].

Bacteria exist across human, animal and environmental niches, and transmission of antibiotic resistance can occur between different ecosystems, such as farms, hospitals, wastewater treatment plants and natural environments. As well, the environment can act as a reservoir of antibiotic resistant determinants (e.g. resistance genes), that are then shared between and across the different niches [3–5].

In the 20th century, the discovery of antibiotics, not only revolutionized modern medicine, by representing the first effective group of drugs for the treatment of infectious diseases, but also by allowing for invasive clinical procedures, ultimately increasing lifespan and saving countless lives. Moreover, the non-therapeutic application of antibiotics in agricultural settings, applied to promote livestock growth and increase food production, also had a major impact in society [6–9].

However, shortly after the introduction of antibiotics, it was realized that bacteria would swiftly develop resistance. The phenomenon was mostly driven by the widespread use of antibiotics and, the increasing rates of antimicrobial resistance, attributed to multiple factors, including but not limited to the continuous overuse of antibiotics and to the lack of new antibiotic drugs in the development pipeline. As well, an important factor contributing to the problem is bacteria ability to acquire and regulate an endless array of resistance mechanisms. Due to this, antimicrobial resistance represents a global crisis of growing concern to human, animal and environmental health [7,10–12].

MECHANISMS AND ACQUISITION OF ANTIBIOTIC RESISTANCE

Mechanisms underlying antibiotic resistance are intricate. Often a single bacteria cell can harbor multiple mechanisms of resistance, contributing to the multidrug resistance phenotype. As well,

resistance to the same antibiotic class may be conferred through different mechanisms. In addition, some bacteria can also have intrinsic resistance to certain antibiotics classes, due to naturally inherit physiological properties (e.g. efflux pumps in Gram-negative), absence of the required antibiotic target and, lack of the metabolic or enzymatic pathways necessary for drug activation [11,13,14].

From an evolutionary and genetic perspectives, two main strategies can be applied by bacteria to acquire antibiotic resistance. These include mutations in genes often associated with the antibiotic mode of action or through the acquisition of mobile genetic elements (MGEs), frequently carrying resistance determinants [3,15].

In the context of mutational resistance, there are four main mechanisms that may lead to antibiotic resistance, including modification of conserved antibiotic targets, up-regulation of active efflux pumps or down-regulation of uptake mechanisms leading to low compound permeability and through metabolic bypass (Figure 1) [13,15].

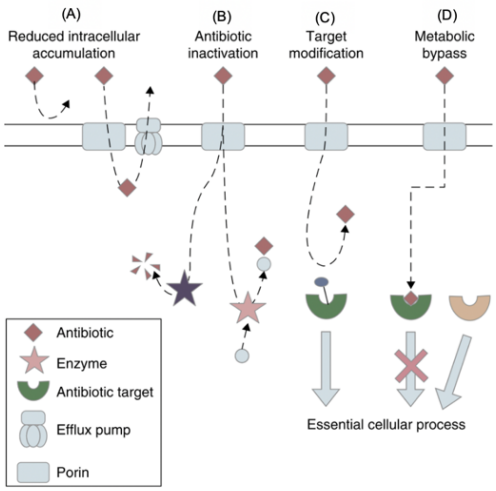

Figure 1. Overview of mutational antibiotic resistance mechanisms (adapted from Bacterial Resistance to Antibiotics – From molecules to man, 2019 [16]). **(A)** Antibiotic resistance may occur by increased efflux or reduced antibiotic permeability leading to decrease of the antibiotic intracellular concentration, **(B)** inactivation of the antibiotic target by enzymatic events or **(C)** by target modification preventing the antibiotic to bind to the target and, finally **(D)** resistance may occur through metabolic bypass.

As mentioned above, bacteria can become resistant through the acquisition of resistance determinants. This may occur by the intake of foreign DNA through events of conjugation,

transformation or transduction, resulting in horizontally acquired resistance [17]. As an example, a well described case is the acquisition of methicillin resistance by *Staphylococcus aureus* (*S. aureus*), mediated by the acquisition of a mobile genetic element, the staphylococcal cassette chromosome *mec* (*SCCmec*). The *SCCmec* carries the *mecA* gene, that in turns encodes an altered penicillin binding protein (PBP2a), with low affinity to β-lactam antibiotics [18–20].

Finally, resistance may occur through vertical evolution, where occurrence of spontaneous mutations after exposure to a chemical compound can lead to mutations in the bacterial genome, that are then transferred to the progeny [14].

On the other hand, in some cases, antibiotic resistance may occur without being related to any genetic alteration; this is termed phenotypic resistance and can be associated with specific biological processes such as cell persistence, growth in biofilms, metabolic state and growth conditions (e.g. nutrients available) [21–23].

EPIDEMIOLOGY OF ANTIBIOTIC RESISTANCE

From an epidemiological perspective, distribution and prevalence of antibiotic resistance can vary depending on geographic location, which in turns might be related with socioeconomic conditions and political regulations concerning antibiotic use implemented by each country [24–26]. An example is the use of antibiotics in livestock and veterinary settings. Whereas the use of antibiotics as growth promoters has been banned in many countries, including in Europe and later in the USA, the practice is still allowed, particularly in low income countries as a way to increase production [27,28].

Nevertheless, and despite measures to control the rise of antimicrobial resistance in these particular settings being constantly updated, the fact that antibiotics continue to be used as a way to control endemic or recurrent infectious diseases within farms, generates concern regarding the transfer of resistant bacteria or pathogenic determinants contributing to resistance, from animals

to humans, via the food chain or by other routes, including close contact with the animals by workers [4,29,30].

Concern is mainly related with resistant bacteria known to cause both disease in humans and in animals or that have zoonotic potential, including *Escherichia coli*, *Klebsiella* spp, *Enterococcus* spp and *Staphylococcus aureus*, in particular methicillin-resistant strains (MRSA) [29].

S. aureus, although considered an ubiquitous microorganism that can be found in the environment and colonizing the skin and mucous membranes of humans and other warm-blooded animals, is an extremely versatile human and veterinary pathogen, responsible for causing not only a wide spectrum of systemic diseases, but also a variety of acute and chronic infections [31–33]. Additionally, is one of the most frequent agents associated with healthcare, community and livestock acquired infections, representing a major public health and epidemiological problem with elevated health costs due to its increased morbidity and mortality [34–36].

S. aureus stability and global spread seem to be related to its ability to rapidly acquire resistance determinants, through horizontal gene transfer of mobile genetic elements (MGEs), and to produce multiple virulence factors that can promote immune evasion, colonization and exacerbate infection, overall facilitating the adaptation to different hosts and the emergence of new resistant clones [34,35,37].

In fact, *S. aureus* is described as having a highly clonal genome and can be organized into different clonal complexes (CC) according to genome content, genetic characteristics and possible clinical manifestations. As well, closely related genotypes can be organized into sequence types (ST), that will be then categorized within an established CC [37–39]. Besides the characterization of dominant lineages and allowing to follow trends in clonal dissemination, this contributes to improve epidemiological characterization of different *S. aureus* isolates, or other bacterial pathogens, allowing to associate patterns of antibiotic resistance, geographical distribution and, hosts and possible host-shifts to specific clones, ultimately contributing for the implementation of control measures and design novel or improve current clinical treatments [30,40–46].

Historical and future perspectives of antibiotic discovery

The term antibiotic was first applied, to describe any natural small molecule, produced by a microorganism exhibiting antagonist effect against other microorganisms. Nowadays, the term has a broader meaning, being also inclusive of semi-synthetic and synthetic molecules. Additionally, antibiotics can be classified based on the ability to inhibit bacterial growth (bacteriostatic) or to kill bacteria (bactericidal), spectrum of activity (narrow or broad spectrum) and mode of action [47–49].

Despite several observations of antagonistic activity amid microorganisms, in particular between bacteria and fungi, in the late 19th century, the first molecules with antimicrobial activity described were synthetic compounds. In this context, the first commercialized antibiotic was Salvarsan, an arsenic based compound, discovered in 1909, by Paul Ehlrich, and directed for the treatment of syphilis caused by *Treponema pallidum* [49–51].

Nonetheless, it was penicillin, discovery by Sir Alexander Fleming in 1928, that kick-started the antibiotic discovery golden era. During this period, a significant number of antibiotic classes, were identified in large systematic screenings of fungal and soil extracts, by employing the Waksman antimicrobial discovery platform [52–54], including streptomycin, the first effective antibiotic against tuberculosis that is still in use today [49]. Due to its success, the platform was widely implemented by the pharmaceutical industry, nevertheless, in the coming years, the interest of mining soil extracts in search for new antibiotics decreased due to the repeated rediscovery of already known molecules [53] .

With this the pharmaceutical industry turned to the *in vitro* semi-synthesis (modification of existing scaffolds or molecular backbones of existing molecules obtained through fermentative procedures) and to the synthesis of molecules, based on the knowledge acquired about the mechanism of action of known antibiotics and, consequent optimization through modification of obtained molecules. For instance, the semi-synthesis of cephalosporines, originally identified as

a metabolite of *Cephalosporium acremonium*, led to the development of several derivative molecules with decreased side effects. As well, an example of a fully synthetic produced antibiotic class are the fluoroquinolones developed in the 60's [1,55–57]. Altogether, said platforms resulted in the major antibiotic classes known today (Table 1) [1,56].

With the genomics era (1995-2004), the ability to identify novel targets, essential for the bacteria survival, by comparing sequenced and annotated genomes from pathogenic and non-pathogenic bacterial strains and to distinguish them from the host, minimizing possible drug-host interactions and reducing possible side effects, redefined the antibiotic discovery paradigm. In consequence, different biochemical target-based screening assays were developed to screen for molecules inhibiting such targets. Although potent inhibitors were identified following this approach, in a whole-cell context, they often failed to reach the target due to low permeability or action of efflux pumps, hampering their activity *in vivo* [1,58]. Additionally, other factors led to the approach limited success, including, but not limited to assays lacking sensitivity for off-target effects. Subsequently, platforms including whole-cell screening, were revived combining the basis of the Waksman platform with genomic knowledge [1,56].

Table 1. Timeline describing the discovery of antibiotic classes combined with mode of action and spectrum of activity [7,49,53].

Antibiotic class and example	Year of discovery	Year of introduction	Year of observed resistance	Activity	Mechanism of action	Target specie(s)
ß-lactam Penicillin	1928	1938	1945	Bactericidal	Inhibition of cell wall biosynthesis	Broad-spectrum
Sulfonamides Prontosil	1932	1936	1942	Bacteriostatic	Inhibition of dihydropteroate synthetase	Gram-positive
Aminoglycosides Streptomycin	1943	1946	1946	Bactericidal	Binding of 30S ribosomal subunit	Broad-spectrum
Tetracyclines Chlortetracycline	1944	1952	1950	Bacteriostatic	Binding of 30S ribosomal subunit	Broad-spectrum
Chloramphenicols Chloramphenicol	1946	1948	1950	Bacteriostatic	Binding of 50S ribosomal subunit	Broad-spectrum
Macrolides Erythromycin	1948	1951	1955	Bacteriostatic	Binding of 50S ribosomal subunit	Broad-spectrum
Polymyxins Colistin	1949	1959*	2017	Bactericidal	Disruption of cell wall	Gram-negative
Glycopeptides Vancomycin	1953	1958	1960	Bactericidal	Inhibition of cell wall biosynthesis	Gram-positive
Oxazolidinones Linezolid	1955	2000	2001	Bacteriostatic	Binding of 50S ribosomal subunit	Gram-positive
Ansamycins Rifampicin	1957	1958	1962	Bactericidal	Inhibition of RNA synthesis	Broad-spectrum
Quinolones Ciprofloxacin	1961	1968	1968	Bactericidal	Inhibition of DNA synthesis	Broad-spectrum
Lipopeptides Daptomycin	1986	2003	2005	Bactericidal	Depolarization of cell membrane	Gram-positive

* First introduction into the clinic; it was later removed due to nephron and neurotoxicity and re-introduced in 1998 as last resort antibiotic.

Nevertheless, despite all efforts for the discovery of novel molecules with antimicrobial properties, since the 60's no new broad-spectrum antibiotic was discovered [49]. Although, colistin, daptomycin and linezolid were introduced in the clinic later, and cases of resistance were already reported [59–61]; colistin, due to its toxicity, is only applicable as a last resort antibiotic for the treatment of infectious diseases caused by multidrug resistant Gram-negative pathogens, whereas daptomycin and linezolid are only relevant for the treatment of infections caused by Gram-positive bacteria [62–64]. As well, since 2009, different derivatives from existing classes were approved by the U.S. Food and Drug Administration (FDA), for the treatment of Gram-positive related infections, including, but not limited to tedizolid and delafloxacin, belonging to the oxazolidinone and fluroquinolone classes, respectively [65].

The fact that the latest antibiotics introduced in the market and new antibiotics in the clinical development pipeline are derivatives of existing classes, can in time become an issue, since there is the possibility of bacteria develop resistance towards them at a faster pace [66,67].

To address this issue, besides the continuous search for novel molecules or secondary metabolites, either from natural sources or synthetic libraries, by combining cell-based methodologies with genome mining [68,69], different approaches are now being taken for the development of alternative antimicrobial therapies [70,71]. These approaches include the development of antibacterial vaccines [72], antibodies and antibody-drug conjugates [73,74], phage therapy [75,76], microbiome modulating therapies [77,78], anti-virulence therapies [79,80], drug combination to increase potency of already existing antibiotics (e.g. efflux pump inhibitors) [81–84] and repurposing existing drugs [85,86]. The drug repurposing topic will be further developed in the following sections.

A recent review identified 407 preclinical antibiotic projects being developed at 314 institutions, including universities and small to medium pharmaceutical companies [87]. An overview of the current preclinical antibacterial pipeline is given in Figure 2.

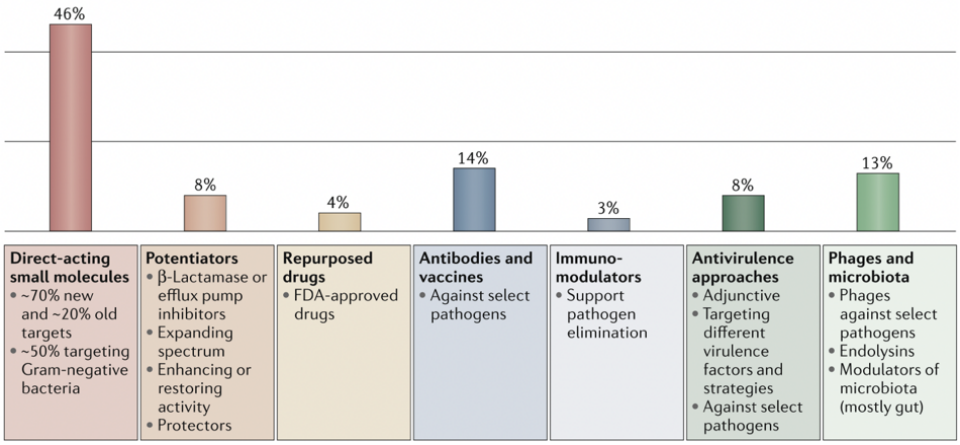

Figure 2. Preclinical pipeline overview. The different approaches are classified in 7 groups, according to their effect on bacteria. The percentages reflect the projects being carried out in each group (Adapted [87]).

Nonetheless, all of these approaches may have high translational risks, related with pharmacokinetics parameters, that may influence administration and/or formulations. As well, other challenges may be presented as it is the case of limited clinical use, since most of these approaches are pathogen-specific [70,87].

THE NEED FOR NOVEL ANTIMICROBIALS

Associated with the lack of novel antimicrobials being approved and reaching the market, multidrug-resistant bacteria continue to emerge, representing not only a public health concern but also a major economic burden, due to the high morbidity and mortality rates associated with infectious diseases [7,88–90]. Because of these issues and to guide the antibiotic drug discovery and development processes, in 2017, the World Health Organization (WHO) published a global priority list reporting the resistant bacteria towards which new antimicrobial treatments are urgently needed [91].

The list, divided in different priority levels, encloses both Gram-positive (e.g. MRSA and vancomycin resistant enterococci) and Gram-negative (e.g. carbapenem-resistant *Acinetobacter baumanni*, *Pseudomonas aeruginosa* and *Enterobacteriaceae*) bacteria, towards which current clinical therapeutic options are becoming scarce due to increased resistance. As well, cases of

resistance were already reported to last resort antibiotics used to treat infectious diseases caused by these bacteria [91,92].

The lack of novel antibiotics, it is particularly worrisome in the case of Gram-negative bacteria, being one of the biggest challenges when searching for new antibiotics, the penetration barrier and efflux mechanisms present in these bacteria [93–98]. In many cases multidrug efflux pumps can confer resistance to entire classes of antibiotics and, although they are expressed in both Gram-positive and Gram-negative bacteria, they are a more potent resistance mechanism in the later one. In particular the clinically relevant *AcrAB-TolC* efflux pump not only mediate intrinsic and acquired resistance but is also involved in the bacterial stress response and pathogenicity [93]. The fact that drugs are almost immediately expelled out of the cell combined with the double cell membrane, that acts as a barrier and lowers drug permeability into the cytoplasm contributes not only to the lack of effective antimicrobial molecules targeting Gram-negative bacteria but also to these bacteria ability to resist the action of structurally diverse compounds [93,98]. These factors highlight the fact that disparities in antibiotic activity between Gram-positive and Gram-negative bacteria are not always related with differences in biological targets, but rather the consequence of additional permeability and efflux barriers possessed by the later one [96,99].

ANTIBIOTIC DRUG DEVELOPMENT PROCESS

Antibiotic drug development is a long and costly process, as it takes several years between the discovery of a compound with antimicrobial properties and its approval by regulatory agencies, such the FDA. Keeping this in mind, when a compound displaying antimicrobial properties is discovered, further steps to evaluate activity, mode of action and safety, are required. These steps are often performed according to specific guidelines of hit to lead and candidate selection, followed by clinical trials (Figure 3) [54,55].

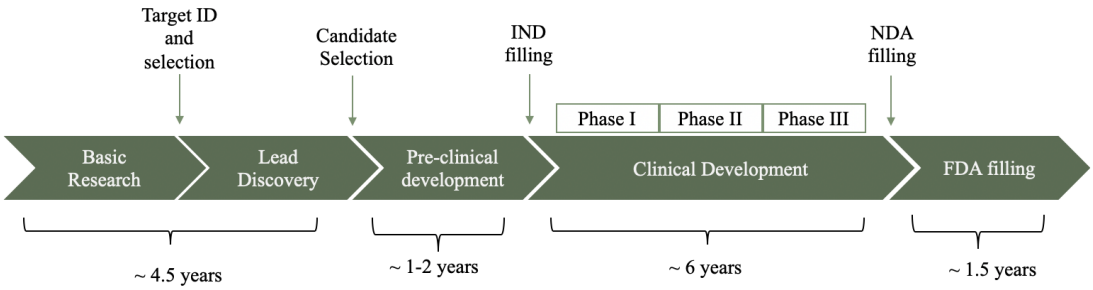

Figure 3. Approximate timeline required from compound identification to FDA approval. IND: Investigational New Drug; NDA: New Drug Application (Adapted [54,100]).

Concept of ideal antimicrobial compound

Although the ideal antibiotic does not exist, based on the knowledge acquired, an ideal antimicrobial compound can be defined as a small molecule, with a broad activity spectrum and the ability to kill or inhibit bacterial growth, regardless the site of infection and without off-target consequences to the host, as it is the case of toxicity. Also, it should have demonstrated a low potential to develop resistance *in vitro*. Moreover, an ideal antimicrobial molecule would be chemically stable and easy to modify, enabling chemical alteration for the purpose of identifying and synthetizing derivative compounds with increased activity and other desirable pharmacokinetic properties, including absorption, distribution, metabolism and excretion (ADME), with the goal of increasing the therapeutic effect while administrating the lowest dose possible [58,101–103].

Target selection

Although many studies start with identification of active molecules, leaving target identification for a later stage. An early step in the antibiotic drug discovery process is usually the identification and validation of a biological target [104].

The ideal drug target should be present in a wide range of bacterial pathogens and have a critical biochemical function within the bacteria cell without pre-existing recorded resistance. Target

identification can be achieved by analyzing genomes of the pathogen of interest, followed by phenotypic cell-based assays for further validation. In the context of searching for novel antimicrobial compounds, one should also consider searching for novel targets, including genes coding for essential functions within a pathogen of interest. To achieve this goal, different approaches can be taken including, transposon mutagenesis and construction of systematic gene-by-gene knock-outs [58,102].

From hit-to-lead to pre-clinical development stage

Regardless the initial approach being target or screen based, in an early screening program, several potential hit compounds might be identified and it is essential to confirm antimicrobial activity and potential liabilities, before selecting and moving forward with the lead compound [58,104]. As a direct consequence, the aim of the hit-to-lead stage is to refine the hits identified, in order to attain the compound(s) with the most desirable activity towards the bacterial specie(s) of interest and that possesses the adequate pharmacokinetics properties, to forward evaluate its activity and effectiveness in *in vivo* relevant models [54] (Figure 4).

As well, if not known before, the structure of the hit compound should be determined, to ensure that is in fact a novel compound (when screening natural compounds libraries) and that there is the possibility of applying for a patent. Knowing the structure will also allow for structure activity relation (SAR) studies. The link between the structural features of a series of molecules and the activity observed may provide knowledge about which chemical groups appear to be responsible for the desirable and non-desirable characteristics, giving room for further optimization [58,104,105].

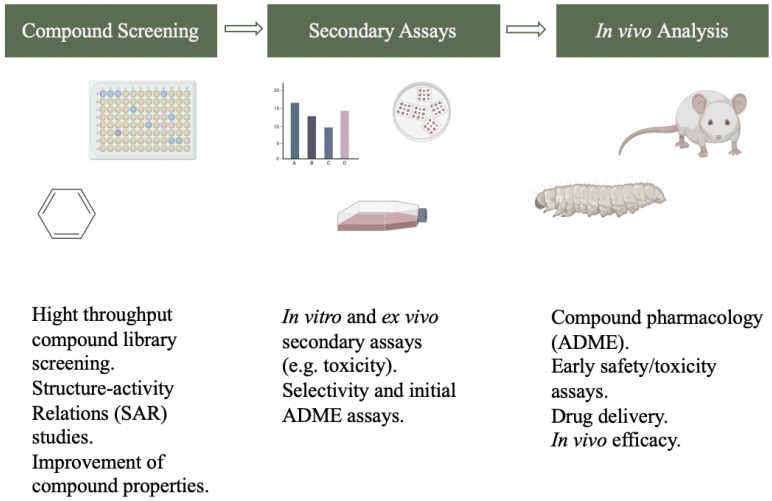

Figure 4. Overview of drug discovery assays from target validation to pre-clinical development (Adapted [54,58]). ADME: absorption, distribution, metabolism and excretion. The figure represents the different steps and assays related to early drug discovery up until pre-clinical development and *in vivo* assays.

Once effectiveness of the lead compound(s) is determined both *in vitro* and *in vivo* and there is no serious liabilities, a candidate drug might be declared ready for entry into a preclinical development program before moving into clinical trials. Although it may vary, experiments conducted at this stage involve the assessment of toxic and genotoxic potential in at least two animal models, extensive characterization of compound efficacy *in vivo* and, formulation testing *in vivo* to guarantee drug delivery to the desired targets [58,106,107].

Clinical development stage

The clinical development stage is often divided in three phases described in Figure 5. Consent to proceed into clinical trials is given after approval of the Investigational New Drug (IND) application submitted to the FDA. In this application, data regarding *in vivo* toxicity and possible side effects, manufacturing information, clinical protocols, any available data from prior human research (e.g. in the cases of drug repurposing this data should be available) and information about the investigator [108].

The different phases will have distinctive purposes, but ultimately the main focus of the clinical trials is to access drug safety in humans. In this context, Phase I and II, are meant to determine

drug efficacy and the maximum tolerable dose that can be administered without trigger major side effects [107]. Besides safety, these phases are usually designed to test pharmacokinetics and pharmacodynamics properties, determine optimal dose and frequency and, administration routes in preparation for Phase III. Finally, Phase III is usually conducted in a larger and often more diverse population, in order to confirm efficacy and to estimate the incidence of common adverse reactions. As well, the comparison of the in study therapy with standard therapies or a placebo is conducted at this stage [107,109].

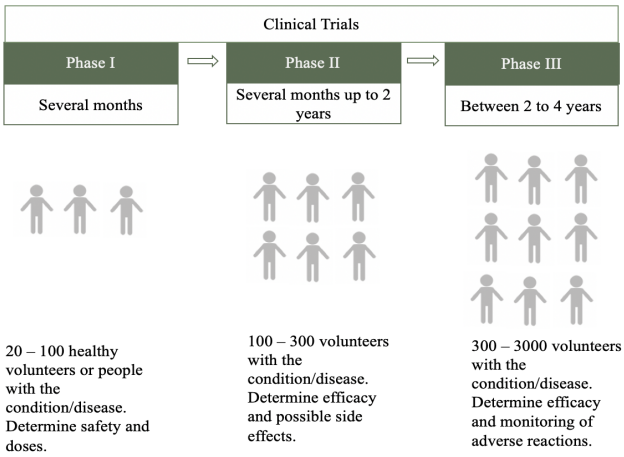

Figure 5. Overview of the clinical trials phases up to FDA approval (information retrieved from the FDA website [108]).

DRUG REPURPOSING

It is becoming clear that the pace of antibiotic drug discovery cannot keep with the increasing emergence of resistant pathogens. In consequence, drug repurposing of already approved drugs outside of their application scope, has emerged as an alternative approach to *de novo* antibiotic drug discovery [110,111].

Several advantages to this strategy can be recognized, including decrease costs and time needed for drug development. As well, data regarding pharmacokinetics, drug safety (e.g. toxicity, potential drug interactions) and preclinical studies are usually available, reducing the risk of failure [112–114].

Both experimental and computational approaches, often in combination, can be taken when starting a repurposing drug campaign. However, in the case of repurposing drugs for treating bacterial infections, frequently the most common approach is the phenotypic screening of a panel of bacteria against the chosen drug collection. Different phenotypic assays are available, including, but not limited to, the determination of the concentration at which the compound is active by measuring the minimal inhibitory concentration (MIC) and cell viability assays (e.g. bioluminescence assays). These assays are usually robust and can be optimized to be applied in a high throughput manner, allowing the screening of large compound collections in a relatively short period of time [79,115–118]. After the active compounds are identified, one can typically move directly to test the compounds in the relevant *in vivo* model, skipping development steps [116].

Despite the described advantages, there are still significant hurdles associated with drug repurposing. A major one is that the therapeutically approved concentration of the identified compound, is usually not potent enough for the new indication, meaning that a higher dose would be needed, which in turn can potentiate undesirable toxicity and other side effects. Additionally, as a result of the higher concentrations needed, the original pharmacokinetic profiles may also be inadequate for the new indication specifically in the case of half-life requirements, tissue distribution and plasma protein binding. The need for clinical trials to design new pharmacokinetics profiles, to address these concerns, together with intellectual property rights, may also represent a major financial throwback to the approach. Nevertheless, drug repurposing can provide new chemical lead scaffolds for the drug discovery process [116,119–121].

REPURPOSING SALICYLANILIDE COMPOUNDS

Salicylanilides (N-substituted hydroxy benzamides) are a large group of organic compounds, with a broad range of biological activities and commercialized for different pharmacological applications [122,123].

Originally developed as antiseptics and fungicides, with the generation of derivative compounds, salicylanilides were later found to be potent anthelmintics. For instance, chlorinated derivatives, such as niclosamide, oxyclozanide and rafoxanide were first commercialized as molluscicidal and anthelminthic agents, to treat parasitic infections in humans and animals. While, brominated derivatives, including tribromsalan and metabromsalan, were used as antimicrobial and antifungal agents in soaps and antiseptics [122,124–127].

Furthermore, salicylanilides, generally consist of highly lipid-soluble weakly acidic phenolic compounds, which basic chemical structure consists of a salicylic acid ring together with an anilide ring (Figure 6). In addition, depending on the type of structural substitution, salicylanilide derivatives can be further classified as halogenated, non-halogenated, acetyl-salicylanilides, dichlorosalicylanilides and benzoylsalicylanilides compounds [122,123]. As well, based on analysis of structure activity relationships (SAR), it was suggested that the broad range of biological activities, displayed by these compounds, it is dependent and varies according to the type of substitution and physicochemical properties of the compounds. For instance, antibacterial activity of derivative compounds was shown to decrease with increase lipophilicity, molar weight and surface activity [128,129].

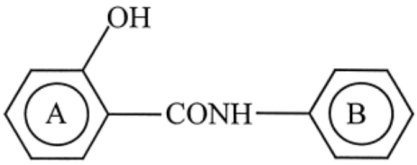

Figure 6. General chemical structure of salicylanilides based compounds. A – salicylic ring and B – anilide ring [122]

In regard to mode of pharmacological action, early *in vitro* studies, using houseflies and rat liver mitochondria, showed several salicylanilides as potent inhibitors of this electron transport associated phosphorylation. In consequence, the primary action of salicylanilides has usually been linked with the uncoupling of oxidative phosphorylation [130].

The interest in repurposing salicylanilides commenced when several studies provided evidence that compounds belonging to this group, in particular niclosamide, were able to interfere with multiple signaling pathways and biological processes. Besides its original application as molluscicidal and as an anthelminthic [126,131], niclosamide was shown to exhibit antimicrobial [132–137], antiviral [138–140], anti-cancer [141,142] and anti-diabetic activities [143]. As a consequence, multiple studies reported the synthesis of a wide range of derivative compounds, in order to improve compounds potency and other pharmacological traits [126,144,153–160,145–152].

NICLOSAMIDE ANTIMICROBIAL PROPERTIES

Niclosamide was discovered in 1953, in the Bayer chemotherapy research laboratories. The compound was originally developed as a molluscicide and marketed under the name Bayluscide in 1959, to kill snails, an intermediate host of schistosomiasis. Shortly thereafter, it was found to be also effective for the treatment of cestode infections (e.g. tapeworm infections), leading to its approval by the FDA and further commercialization for human use [127,161]. Since then, niclosamide is included in the WHO list of essential medicines and has been used to safely treat parasitic infections in humans [162].

Niclosamide, is chlorinated salicylanilide compound, that can be included in the halogenates group. It consists of a small lipophilic molecule with low aqueous solubility (Figure 7) [127].

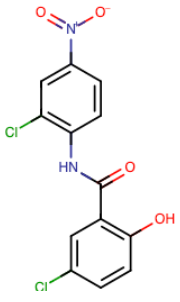

Figure 7. Niclosamide chemical structure ($C_{13}H_8Cl_2N_2O_4$) [163].

Despite being extensively used for the past 60 years, the exact mechanism of action of niclosamide has not yet been fully understood. Nevertheless, it is believed that niclosamide functions as a hydrogen ionophore and that anthelminthic activity is related with the uncoupling of mitochondrial oxidative phosphorylation from electron transfer which in turns inhibits adenosine triphosphate production, leading to tapeworm death [130,137,164].

It is hypothesized that niclosamide displays a similar mode of action towards bacteria, by functioning as a protonophore and dissipating transmembrane potential or the proton motive force (PMF). The PMF is the electrochemical gradient of protons generated across the cell membrane, resulting from the extrusion of protons by the electron transport chain. The PMF is necessary for ATP synthesis and several ATP dependent processes, including flagellar motility, import of nutrients and transport of multiple solutes. The dissipation of the PMF leads to inhibition of these important functions and consequently macromolecular biosynthesis stops. Accordingly, cell growth is affected and loss of bacterial viability occurs [128,132,136,165].

As stated before, niclosamide was shown to have a broad range of biological activities, including antimicrobial activity. In this context, niclosamide was shown to be highly effective *in vitro* towards *Mycobacterium*, several Gram-positive bacteria, including MRSA clinical isolates and Gram-negative *Helicobacter pylori* (Table 2). Infections caused or exacerbated by these bacteria represent an ever-increasing therapeutic challenge [98]. Current research, also demonstrates that niclosamide is effective at concentrations lower than the conventional antibiotics used in the clinical practice, such as vancomycin, for the treatment of MRSA, enterococci and *Clostridium* related infections [132,135,166] plus, clarithromycin and metronidazole used to treat *H. pylori* infections [167,168]. In addition, although niclosamide has been extensively used for decades, resistance was not yet reported, which increases its potential as an alternative to conventional antibiotics [137].

Table 2. Niclosamide antimicrobial activity towards clinical important bacteria.

Bacteria	MIC range (μg/mL)	Studies
Mycobacterium tuberculosis *Mycobacterium bovis*	0.5 – 1	133,169–171
MRSA	≤ 0.0625 – 0.5	132,137
Staphylococcus epidermidis	0.5	132,137
Enterococcus faecium *Enterococcus faecallis*	0.25 - > 128	132,135
Clostridium difficile	0.5 – 4	134,172
Helicobacter pylori	0.25	136

MIC: minimal inhibitory concentration.

As well, niclosamide was shown to both prevent biofilm formation and to inhibit mature biofilms, revealing a broad anti-biofilm activity against MRSA and *S. epidermidis* biofilms, at concentrations comparable to the MIC [137,173].

Furthermore, niclosamide was shown to be bacteriostatic against MRSA, *E. faecium* and *H. pylori* [132,135,136] and bactericidal towards logarithmic and stationary phase *C. difficile* [134].

Besides *H. pylori*, niclosamide is not particularly active towards Gram-negative bacteria, likely due to the action of efflux transporters or other functional and structural characteristics common to this group of bacteria [15,132]. In fact, recently the lack antibiotic activity of niclosamide towards *E. coli* was associated not only with the action of multidrug efflux pumps, but also with a mechanism of nitroreduction [174]. Interestingly, niclosamide was reported to inhibit *Pseudomonas aeruginosa* quorum-sensing at micromolar concentrations, leading to the inhibition of several secreted virulence factors, including pyocyanin and elastase, and exposing its potential to act as an indirect inhibitor of Gram-negative pathogenesis [175]. Moreover, niclosamide was reported to potentiate colistin activity towards colistin-resistant *Klebsiella pneumoniae* and *Acinetobacter baumanni* [81].

A limiting aspect about repurposing niclosamide as an antibiotic, is related to its low absorption and poor oral bioavailability, characteristic that seems to be common to the class. Such trait may render it ineffective for treating systemic infections. In fact, early research indicated that niclosamide was only partially absorbed in the gastro-intestinal (GI) tract, with a maximum serum concentration below 6 μg/mL, after oral administration to healthy volunteers. As well, it was

found to be poorly distributed in the tissues and highly biding to plasma proteins, characteristics also contributing to a low systemic effect [127]. Nevertheless, these characteristics make of niclosamide a desirable decolonization agent to reduce for instance, enterococci, *H. pylori* or *Clostridium* in the GI tract. Because of its poor absorption it is hypothesized that, niclosamide would accumulate at a sufficient concentration in the gut, to exert a therapeutic effect [134–136].

Another possible indication for niclosamide, that will be further discussed in Manuscript I, it is its topical application in atopic dermatitis lesions as a decolonization agent against *S. aureus*, that it is described to exacerbate these lesions. Current therapeutic options, that include fusidic acid and mupirocin, are becoming ineffective due to resistance, leading to treatment failure and enhancing the need for new therapies with a reduced likelihood to develop resistance [176–178].

CHAPTER II – MANUSCRIPT I

ATx201 REDUCES *STAPHYLOCOCCUS AUREUS* COLONIZATION AND INCREASES SHANNON DIVERSITY OF THE SKIN MICROBIOME IN ATOPIC DERMATITIS PATIENTS

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One sentence summary

ATx201 has a novel mode of action against *S. aureus* demonstrating reduction of *S. aureus* colonization in patients with mild-to-severe atopic dermatitis without promoting evolution of resistance.

Abstract

Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by a complex interplay between a compromised epidermal barrier and an abnormal immune response. *Staphylococcus aureus* frequently colonizes AD lesions and is linked to disease severity and believed to contribute to disease progression. To better address the microbial component of AD there is a need for treatments that reduce *S. aureus* colonization without significantly affecting the skin commensal microbiota. We describe ATx201, which targets the proton motive force and acidifies the cytosol of *S. aureus* effectively stopping cell growth. ATx201 has a narrow MIC distribution (0.125 - 0.5 μg/mL) consistent with its mode of action. In murine models, ATx201 can effectively treat superficial skin infections of methicillin-resistant *S. aureus* (MRSA). In a randomized, double-blind and vehicle-controlled trial in patients (n = 36) with mild-to-severe AD we found that ATx201 OINTMENT 2% effectively reduces *S. aureus* colonization while increasing the Shannon diversity of the skin microbiome. These results suggest that ATx201 OINTMENT could become a new treatment modality for AD patients.

Introduction

Atopic Dermatitis (AD) is a chronic inflammatory disease with a prevalence of 2-10% in adults in developed countries [1]. AD is a multifactorial disease with a complex interplay of immunological mechanisms, predominantly Th2/Th22 immune responses, and an altered epidermal skin barrier, characterized by trans-epidermal water loss and epidermal hyperplasia [2, 3]. Patients with AD are prone to bacterial colonization by *Staphylococcus aureus* on both lesional

and non-lesional skin [4–6]. Indeed, more than 90% of patients with AD are colonized with *S. aureus* and the density of *S. aureus* on acutely inflamed AD lesions is generally more than 1000-fold higher than on non-lesional AD skin and peaks during disease flares [5, 7]. The greater *S. aureus* colonization and proliferation probably results from a combination of a compromised functional epidermal barrier with reduced lipid content, increased adherence potential because of augmented fibronectin and fibrinogen in the stratum corneum, diminished immune recognition and impaired antimicrobial peptide production in patients with AD [8–12].

In turn, *S. aureus* colonization is a significant factor in the pathogenesis of AD, leading to exacerbation of the disease [5, 11]. *S. aureus* secretes toxins, that act as super-antigens and exogenous protease inhibitors that further damage the epidermal barrier and potentiate allergen penetration that trigger multiple inflammatory reactions even in absence of overt infection [11]. *S. aureus* enterotoxins provoke the generation of enterotoxin specific IgE and thereby increase inflammation in AD [13]. These enterotoxins interact directly with MHC II and the beta chain of the T cell receptor to induce an antigen-independent proliferation of majorly type 2 T cells [13]. Secretion of alpha toxin, a destructive pore-forming toxin often found in the lesions of patients with severe AD, is primarily associated with keratinocyte death, which in turn correlates to increased exposure to Th2 cytokines [14–16]. Expression of delta toxin by *S. aureus*, a phenol-soluble modulin peptide, has been described to cause lysis-independent mast cell degranulation and was also found in lesions of AD patients [6, 9].

In recent studies, *S. aureus* relative abundance was further linked to the skin microbiome dysbiosis in patients with AD. Shifts in microbial diversity and load of *Staphylococcus* are correlated to specific AD disease states and disease progression. The abundance of *Staphylococci*, specifically *S. aureus*, was shown to be greater and the Shannon diversity lower during disease flare compared to baseline or post-treatment/resolving flares [7]. Thus, *S. aureus* and the skin microbiome represent a promising target for the treatment of AD by addressing the “outside-in” mechanism of the disease [17]. Indeed, antibiotics such as fusidic acid or mupirocin are commonly

prescribed to AD patients to address the microbial component of the disease. Unfortunately, these antibiotics exhibit major drawbacks. Worldwide resistance rates towards fusidic acid and mupirocin are high in dermatology patients with reported rates up to 41% and 47%, respectively [18–20] and resistance towards fusidic acid evolves rapidly during treatment [21].

To exploit recent insights regarding the importance of *S. aureus* in AD, microbiome therapies are emerging in the clinic with the objective to reduce the abundance of *S. aureus* and increase microbial diversity by introducing one or more beneficial commensal strains. It was reported that transplantation of *Staphylococcus hominis* and *Staphylococcus epidermidis* strains reduced the bacterial load of *S. aureus* on the skin of AD patients, through exerting antimicrobial activity towards *S. aureus* [22]. Another study demonstrated that *Bacillus subtilis* lipopetides eliminate *S. aureus* by inhibiting its quorum-sensing ability [23]. The presence of Esp-secreting *S. epidermidis* was also shown to eliminate the nasal colonization of *S. aureus* by diminishing the biofilm formation of *S. aureus* [24]. While therapies based on addition of living bacteria represent a promising therapeutic approach, challenges include the affordable manufacturing, formulation and long-term stability of such therapeutics. Furthermore, it remains unclear whether the use of transplants of specific commensal bacteria can eliminate resistant strains on the human AD skin, such as methicillin-resistant *S. aureus* (MRSA). Thus, a small molecule with a narrow-spectrum effect, including *S. aureus*, could represent an attractive alternative for the treatment of AD.

In this study we examined the potency and the spectrum of ATx201 in pre-clinical models and further analyzed its propensity for resistance evolution. The impact of ATx201 OINTMENT 2% on the skin microbiome and load of *S. aureus* was then assessed in a clinical trial in patients with mild-to-severe AD.

Results

ATx201 is a potent agent against *S. aureus* overcoming existing resistance mechanisms with dose dependent kill kinetics

ATx201 was identified in a previous high-throughput screen as a compound with activity against MRSA USA300 [25, 26]. To assess the breadth of effect of ATx201 against a broad panel of clinical isolates, 206 clinically relevant *S. aureus* strains, including both MRSA (n = 118) and methicillin-sensitive (n = 88) strains, were screened. The panel included strains resistant to commercialized comparators commonly used for the topical treatment of skin infections, such as fusidic acid (n = 60), mupirocin (n = 3), retapamulin (n = 1), and clindamycin (n = 13). The minimal inhibitory concentration (MIC) of ATx201 ranged from 0.125 to 0.5 µg/mL against all *S. aureus* strains regardless of their resistance towards the comparators (Figure 1A, Table S1). The remarkable consistency in the MIC against diverse isolates of *S. aureus* suggests that growth might be inhibited through a novel mechanism of action.

To test whether ATx201 has a bacteriostatic or bactericidal effect, time-kill curves against both methicillin resistant (MRSA 43484 = MRSA 01) and susceptible (RN422) strains of *S. aureus* were determined. ATx201 MICs were estimated after 18 hours of incubation and were identical for both strains (Table S1). ATx201 exhibited bacteriostatic killing kinetics at 1× and 1.5×MIC, corresponding to 0.25 µg/mL and 0.375 µg/mL, respectively. However, at 4×MIC (1 µg/mL) ATx201 cleared the bacterial populations of RN422 and MRSA 43484 after 48 and 72 hours, respectively (Figure 1B).

ATx201 arrests cell growth and inhibits specific biosynthetic pathways

To evaluate the kinetics of ATx201, the bacterial growth inhibition of exponentially growing cultures of *S. aureus* ATCC 29213 were treated with 1×, 4× or 10× MIC of ATx201 or known standard DNA, RNA, protein and cell wall synthesis inhibitors ciprofloxacin, rifampicin, erythromycin and vancomycin, respectively. Optical density at 600 nm was quantified over time

for these different drug exposures. Immediate growth arrest was observed upon addition of 1×MIC ATx201 (Figure S1). In contrast, none of the comparators arrested cell growth immediately at 1×MIC. Cell growth inhibition by 1x to 10× MIC ATx201 did not cause cell lysis. To further investigate the mechanism of action, the effect of ATx201 on macromolecular biosynthetic activity was characterized. The rate of DNA, RNA, protein and cell wall biosynthesis was studied using corresponding radiolabeled precursors along with a well characterized antibiotic inhibiting each corresponding biosynthetic activity. We found that ATx201 strongly inhibits all biosynthetic activities tested even with a higher efficiency and at lower concentrations compared to the relevant specific antibiotic inhibitors (Figure 1C). This data suggests that ATx201 rapidly inhibits cellular metabolism upstream of the specific biosynthetic processes. In addition to the specific antibiotic inhibitors, we also tested carbonyl cyanide m-chlorophenyl hydrazone (CCCP), a compound known to depolarize the cell membrane of bacteria. Notably, CCCP displayed a similar inhibition of the biosynthetic activity of the cell to ATx201, suggesting a similar mode of action (Figure 1C).

ATx201 dissipates the proton motive force and acts as a proton carrier

To study the impact of ATx201 on the proton motive force in *S. aureus* we deployed a membrane specific fluorescence dye 3,3'-dipropylthiadicarbocyanine iodide (DiSC3(5)), which localizes in the bacterial membrane in a proton motive force dependent manner such that its fluorescence increases upon dissipation of the proton motive force [27]. We found that DiSC3(5) fluorescence increased upon treatment of DiSC3(5) labelled *S. aureus* ATCC 29213 or MRSA 43484 with ATx201 (Figure 1D). A similar observation was made when DiSC3(5) labelled cells were treated with CCCP. No change of fluorescence was observed upon treatment with equal volume of DMSO solvent or comparator antibiotics, such as vancomycin, erythromycin, rifampicin or ciprofloxacin. These observations suggest that ATx201 dissipates the proton motive force in *S. aureus*, including in MRSA strains, which is in alignment with previous studies [28].

Furthermore, to investigate the potential protonophore activity of ATx201 we used the dye 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM), which is hydrolyzed by cytoplasmic esterases to the BCECF fluorophore [29] and whose fluorescence depends on the cytoplasmic pH. Under normal conditions the cytoplasmic pH lies within the range of 7.4 – 7.8 resulting in high BCECF fluorescence. However, the fluorescence of BCECF is reduced upon protonophore treatment due to influx of protons acidifying the cytosol [30]. Indeed, treatment of BCECF labelled *S. aureus* ATCC 29213 cells with 20 µg/mL nigericin, a protonophore H+/K+ anti-porter, reduced BCECF fluorescence (Figure 1E and Figure S2). Also, a similar drop of fluorescence was observed when BCECF labelled *S. aureus* ATCC 29213 cells were treated with 20 µM of the protonophore CCCP. Treatment of BCECF labelled cells with 0.25 µg/mL ATx201 also reduced BCECF fluorescence. Notably, the cytoplasmic acidification action of 20 µg/mL nigericin and 20 µM CCCP were close and comparable to 0.25 µg/mL ATx201 (Figure 1E). No change of BCECF was observed when labelled cells were treated with 20 µg/mL valinomycin, a K+ ionophore.

If ATx201 acts as a protonophore on *S. aureus* one would expect its potency to increase at acidic pH conditions. Indeed, it was found that the MIC of ATx201 decreased with decreasing pH (Figure 1F), which is in agreement with the hypothesis that ATx201 is a proton carrier that dissipates the PMF through acidification of the bacterial cytosol. Furthermore, it should be noted that the pH range of human skin is between 5 and 6, suggesting that ATx201 inhibitory activity is potentiated relative to the conditions normally used to determine MICs *in vitro*.

Overall, these observations suggest that ATx201 dissipates the proton motive force in *S. aureus*, including in MRSA strains, by reducing the cytoplasmic pH through a proton carrier activity. Currently no approved antimicrobial agents are known to function via this mode of action; accordingly, these data are consistent with ATx201 having a new mode of action.

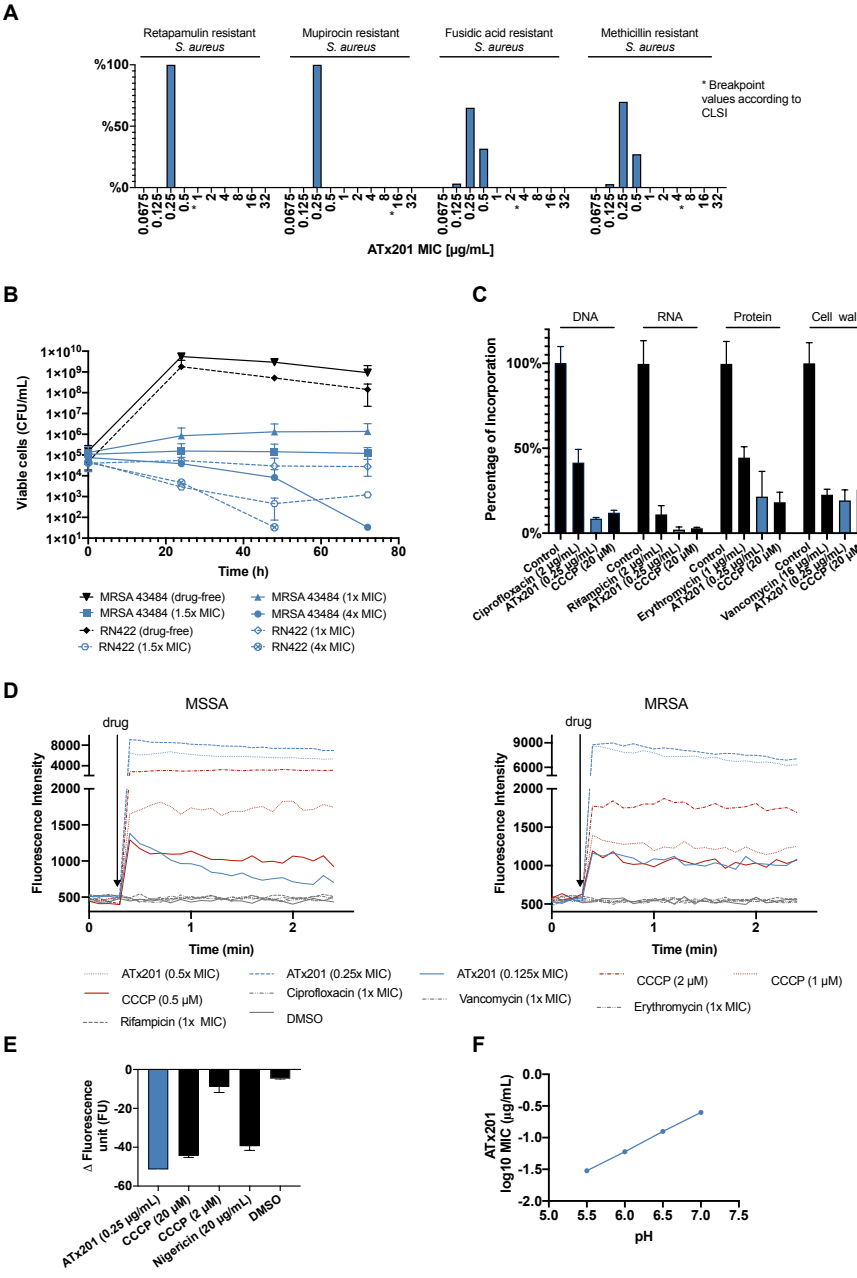

Figure 1. Time-Kill kinetics of ATx201 and its activity to dissipate the proton-motif force (PMF) of *S. aureus.*

A MIC distribution of ATx201 against all tested *S. aureus* strains depending of their resistance profile. **B** ATx201 killing kinetics for MRSA 43484 and *S. aureus* strain RN4220 in drug-free MHB or MHB with different concentrations of ATx201. **C** Effect of ciprofloxacin, rifampicin, erythromycin, vancomycin, CCCP and ATx201 on macromolecule synthesis is presented by percentage of incorporated DNA, RNA, protein and cell wall precursors relative to DMSO treated controls. Average percentage value of each treatment is presented with standard deviation. Antimicrobials with treatment concentration are labelled below the column of corresponding percentage value. DNA, RNA, protein and cell wall synthesis panels are designated above the columns by horizontal lines. Different fold-MIC concentrations were used for different antibiotics given their MIC against MRSA strains. **D** Effect of ATx201, CCCP, DMSO or ciprofloxacin, rifampicin, erythromycin, vancomycin treatments on DiSC3(5) fluorescence in ATCC 29213 (left panel) and MRSA 43484 (right panel) are plotted as a function of time. 1×MIC of ciprofloxacin, rifampicin, erythromycin, vancomycin and ATx201 are 0.25, 0.003, 0.5, 2 and 0.25 µg/mL, respectively. **E** BCECF fluorescence change upon ATx201, nigericin, CCCP or DMSO treatments. **F** Log10 MIC (µg/mL) of ATx201 and its dependence on pH.

S. aureus mutants resistant to ATx201 cannot be selected in the laboratory

A key consideration for the evaluation of the usefulness of any decolonizing agent is the emergence of single-step mutations conferring resistance towards that agent. To assess the likelihood of emergence of spontaneous resistance mutations, a fluctuation assay was conducted for ATx201 and relevant comparators such as fusidic acid, mupirocin, retapamulin and rifampicin, using *S. aureus* MRSA 43484. At a concentration of 4×MIC, we were unable to isolate any resistant mutants towards ATx201. In contrast, we found resistant mutants at 4×MIC for all other tested comparators. The frequencies of resistant mutants of strain 43484 at 4×MIC are 2.00×10–8, 7.90×10–8, 8.02×10–7 and 5.66×10–9 for mupirocin, retapamulin, fusidic acid and rifampicin, respectively (Table 1 and Table 2). These numbers are in agreement with previously published works [31, 32] and correspond to 4×MIC frequency values at least 1000 times higher than that of ATx201 at a concentration of 1×MIC (F = 1.67×10–11).

To further investigate the propensity of *S. aureus* to evolve resistance towards ATx201, we conducted a 40-day serial passage experiment using two MRSA strains (MRSA 16115 and MRSA 38127). Following the 40-day passaging, individual colonies were picked from the evolved populations and the MIC towards ATx201 was determined. Notably, the MIC towards ATx201 remained < 0.5 µg/mL for both MRSA 16115 and MRSA 38127 (Table 3), which additionally supports the low likelihood of resistance evolution of ATx201. Thus, these data highlight that the supply of mutations conferring resistance towards ATx201 is very low compared to currently used cutaneous antimicrobial agents [33].

Table 1. Mutation rates (μ) and frequencies of resistant mutants (*F*) for rifampicin in *S. aureus* strains.

Strain	Rifampicin (16 μg/mL)		
	F	μ	95% CI
MRSA 43484	4.28×10^{-8}	5.66×10^{-9}	$4.89–6.54\times10^{-9}$
RN4220	1.28×10^{-8}	1.39×10^{-9}	$1.60–1.82\times10^{-9}$
RN4220 Δ*mutS*	4.94×10^{-7}	5.11×10^{-8}	$4.72–5.55\times10^{-8}$

Table 2. Mutation rates (μ) and frequencies of resistant mutants (*F*) for ATx201 and other drugs in MRSA 43484.

	F	μ	95% CI
ATx201 (1× MIC=0.25 μg/mL)	1.67×10^{-11}	6.51×10^{-12}	9.18×10^{-13}–4.62×10^{-11}
ATx201 (1.5× MIC=0.375 μg/mL)	< DL*	< DL	–
ATx201 (4× MIC=1 μg/mL)	< DL	< DL	–
Mupirocin (4× MIC=1 μg/mL)	2.00×10^{-8}	2.46×10^{-9}	$1.98–3.06\times10^{-9}$
Retapamulin (4× MIC=0.256 μg/mL)	7.90×10^{-8}	7.50×10^{-9}	$6.46–8.72\times10^{-9}$
Fusidic acid (4× MIC=0.256 μg/mL)	8.02×10^{-7}	8.98×10^{-8}	$8.45–9.55\times10^{-8}$

*At ATx201 concentrations of 1.5× and 4×MIC, the general lack of growth indicates a very low frequency of ATx201 resistant mutants (no colonies were observed on the plates), below the detection limit (DL) of our experimental set up, which could potentially detect 1 mutant in $\sim10^9$ bacteria (corresponding to 1 ml of culture).

Table 3. MICs of ATx201 for *S. aureus* strains exposed to 40 passages of sub-inhibitory concentrations.

Strains	ATx201				
	Parental strains	After 10 passages	After 20 passages	After 30 passages	After 40 passages
ATCC 29213	0.25	0.125	0.25	0.25	0.125
MRSA 16115	0.06	0.06	0.06	0.06	0.06
MRSA 38127	0.125	0.125	0.125	0.25	0.25

ATx201 is effective *in vivo* against MRSA strains

To test ATx201 efficacy *in vivo*, a superficial skin infection model in BALB/c mice was developed [34]. The model was prepared with three different MRSA strains, covering different resistance profiles, including resistance to fusidic acid and mupirocin. Experimental cutaneous formulations of ATx201, as well as commercial cutaneous formulations of fusidic acid and mupirocin, were applied twice-daily for three days. We found that ATx201 significantly reduced the bacterial load of MRSA strains, including both fusidic acid and mupirocin resistant isolates. Treatment with ATx201 (4% w/w) against the type USA300 MRSA 43484 was equivalent to commercial fusidic acid and mupirocin formulations (Figure 2A). However, treatment with ATx201 (4% w/w) against the fusidic acid resistant isolate MRSA 88779 (MRSA 14) was superior to fusidic acid ($P < 0.0001$, ANOVA). Indeed, treatment with fusidic acid did not result

in a significant reduction in MRSA 88779 load compared to the untreated mice (Figure 2B). ATx201 was also able to significantly reduce the bacterial load of the mupirocin resistant MRSA strain 86446 (Figure 2C). Surprisingly, despite an MIC of >256 µg/mL the mean bacterial load of MRSA 86466 was also significantly reduced by 13.8-fold following treatment with 2% w/w mupirocin ($P < 0.05$, ANOVA). However, ATx201 (4% w/w) showed a statistically significant reduction in bacterial load compared to treatment with the commercial mupirocin cutaneous formulation ($P < 0.0001$). In conclusion, these data demonstrate that cutaneous use of ATx201 is as effective as both fusidic acid and mupirocin when treating strains that are susceptible to fusidic acid or mupirocin. However, ATx201 displays a superior efficacy compared to the commercial formulations of fusidic acid and mupirocin when treating strains that are multidrug resistant.

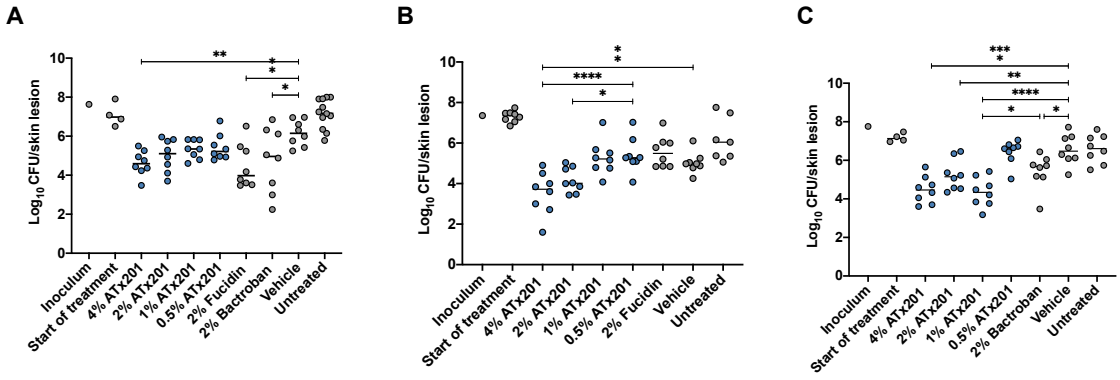

Figure 2. CFUs in the mouse superficial skin wound infection model. Mice were treated twice-daily for three days with vehicle only, an ATx201 dose range or licensed products Fucidin or Bactroban following inoculation with **A** MRSA 43484 (FUS and MUP susceptible) **B** MRSA 88779 (FUS resistant) or **C** MRSA 86446 (MUP resistant). The data is representative of five (**A**) or two (**B** & **C**) independent experiments. **** = $p < 0.0001$, *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$, Dunnett's multiple comparison test.

ATx201 OINTMENT 2% is well-tolerated with no safety issues reported in patients with AD

Following a Phase 1 study in healthy volunteers (data not shown), in which three different cutaneous formulations of ATx201 were tested, the ATx201 OINTMENT 2% formulation was selected for further clinical testing. We studied the safety and efficacy of ATx201 OINTMENT

2% in patients with AD in a randomized, placebo-controlled, double-blinded, split-body designed Phase 2 study (study design displayed in Figure S3). Out of 43 patients that were randomized to ATx201 OINTMENT 2% once-daily or twice-daily and matching vehicle, 36 patients were included in the full analysis set (18 per treatment group). As prospectively defined in the inclusion criteria, seven patients were discontinued on Day 1, due to *S. aureus* colony count below 1,000 CFU/cm^2. Baseline characteristics and demographics were similar across the two dosage groups, once-daily and twice-daily (Table S2). Most patients had a Baseline Investigator Global Assessment (IGA) score of 3 (58.3% in ATx201 OINTMENT 2% group, 55.6% in vehicle group). The mean Baseline Eczema Area and Severity Index (EASI) score was 7.81 in the ATx201 OINTMENT 2% group and 7.86 in the vehicle group, the mean Baseline Visual Analogue Scale (VAS) Pruritus score was 4.52 and 4.5, respectively.

ATx201 OINTMENT 2% was well-tolerated in both dosage groups as no safety issues were identified (overview of AEs in Table S3). All systemic adverse events reported in Table S3 were rated as "not" or "unlikely" related to the study treatment. In contrast, only few local reactions were observed which all were graded as "possibly", "probably" or "related". Local reactions were reported to be "mild" or "moderate" and all fully recovered without need for interruption of treatment. This safety profile is in alignment with previous clinical trials conducted in healthy volunteers demonstrating ATx201 OINTMENT 2% as a well-tolerated agent without any signs of irritation or sensitization at the site of administration and neglectable systemic exposure [35]. ATx201 has been described as a mild mitochondrial uncoupler in human cells with a good safety profile, as mild uncoupling is tolerable in normal cells [36, 37]. This dermatological safety and tolerability is further supported by a clinical study showing that topical application of ATx201 for 6 months did not result in side effects, as well as a murine study in which subcutaneous injection of ATx201 did not cause significant skin alterations in healthy mice [38, 39]. Treatment with ATx201 OINTMENT 2% for seven days improved the clinical scores (EASI, IGA and VAS)

of the treated AD lesion, although not with significant difference between active and vehicle in neither of the two dosage groups.

ATx201 effectively reduces *S. aureus* abundance in AD lesions

The primary efficacy endpoint in this study was eradication of *S. aureus* defined as a >100-fold reduction in the *S. aureus* CFU/cm^2 of sampled skin lesion on day 7 in the ATx201 OINTMENT 2% group compared to vehicle. For the once-daily group, treatment with ATx201 OINTMENT 2% resulted in treatment success and 9 out of 18 patients (50%) at day 7, whereas vehicle showed a 33.3% (6 out of 18 patients) success rate, respectively. For the twice-daily group, treatment with ATx201 OINTMENT 2% resulted in treatment success for 17 out of 18 patients (94.4%) at day 7, whereas vehicle demonstrated a 38.9% (7 out of 18 patients) success rate, respectively. The treatment success was significantly higher for active compared to vehicle at day 7 only in the twice-daily group (Chi-squared test, $P = 0.00157$), but not in the once-daily group (Chi-squared test, $P = 0.18$) (Figure 3). Untreated areas did not show a successful response in any dosage group at day 7 (2 out of 18 patients (11.1%), combined dosage groups). Thus, treatment with ATx201 OINTMENT 2% twice-daily for seven days effectively reduces *S. aureus* in skin lesions of AD patients.

Furthermore, to investigate whether ATx201 would select for resistant mutants in “real-settings”, we selected 26 samples from 36 patients that had cultivatable *S. aureus* isolates at Day 7. We determined the MIC for all isolates and did not observe any strains isolated on Day 7 with increased MICs (> 0.5 µg/mL) towards ATx201 (Table 4). These data along with the *in vitro* assessments of resistance evolution support the hypothesis that ATx201 has a very low propensity to select for resistance.

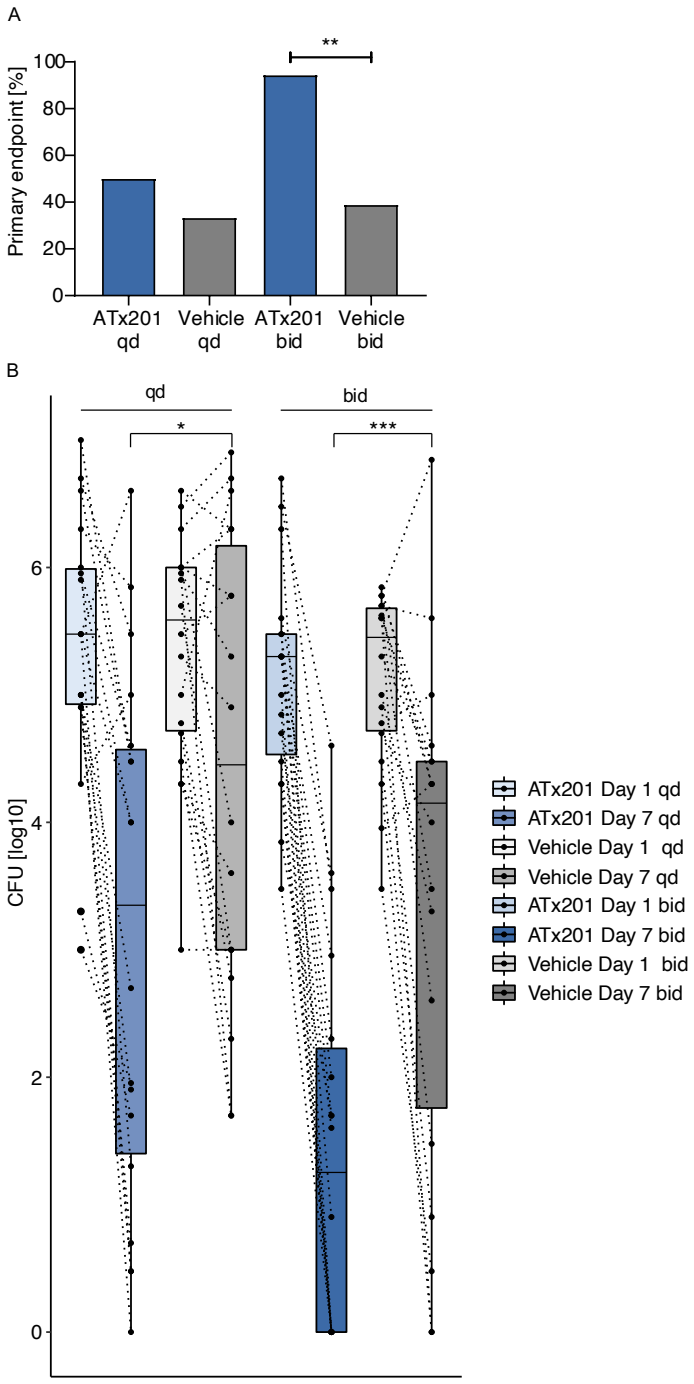

Figure 3. Impact of ATx201 OINTMENT 2% as a decolonizing agent in AD patients.

A Share of patients achieving treatment success at day 7 receiving ATx201 OINTMENT 2% (ATx201) compared to vehicle, defined as a 100-fold reduction in the *S. aureus* CFU/cm^2 per treatment arm. ** = $p < 0.01$, Chi-squared test. N = 18 per dosage group (split-body design). **B** Change in abundance levels (CFUs) of *S. aureus* per dosage and treatment group.

Paired samples are connected with a dotted line. *** = $p < 0.001$, * = $p < 0.05$, Wilcoxon test. Bid: twice-daily, qd: once-daily.

Table 4. MICs of ATx201 in culturable strains isolated from patients at Day 7.

Isolate Day	Subject	Dose	MIC [µg/mL]
D7	C01	bid	0.06
D7	C02	qd	0.06
D7	C03	qd	0.25
D7	C04	bid	0.06
D7	C05	bid	0.125
D7	C06	qd	0.25
D7	C08	bid	0.5
D7	C11	qd	0.25
D7	C12	qd	0.06
D7	C13	bid	0.5
D7	C14	qd	0.125
D7	C16	qd	0.25
D7	C17	bid	0.5
D7	C18	qd	0.25
D7	C20	qd	0.5
D7	C22	qd	0.125
D7	C23	bid	0.25
D7	C26	qd	0.25
D7	C27	bid	0.25
D7	C28	bid	0.25
D7	C30	qd	0.25
D7	C32	qd	0.06
D7	C34	qd	0.25
D7	C38	qd	0.25
D7	C39	qd	0.125
D7	C43	bid	0.125
QC	ATCC 29213	-	0.125 – 0.25

Bid: twice-daily; qd: once-daily; QC: quality control.

ATx201 OINTMENT 2% increases the Shannon diversity of the skin microbiome of AD patients

To further assess the effect of ATx201 OINTMENT 2% on the skin microbiome, the α- and β-diversity of ATx201 was investigated relative to vehicle using 16S rDNA sequencing. The α-diversity of the samples was assessed by counting the number of Operational Taxonomic Units (OTUs) and calculating the Shannon index. The α-diversity of the lesions treated with ATx201

OINTMENT 2% and vehicle were similar at baseline for the once-daily (Wilcoxon test, P_{OTUs} = 0.3517, $P_{Shannon}$ = 0.5619) and twice-daily treatment group (Wilcoxon test, P_{OTUs} = 0.7422, $P_{Shannon}$ = 0.5469). Treatment with ATx201 OINTMENT 2% for 7 days twice-daily resulted in a significant increase of the α-diversity relative to vehicle which was reflected in a significantly higher number of OTUs and a significantly increased Shannon Index relative to vehicle (Figure 4; Wilcoxon test, P_{OTUs} = 0.0142, $P_{Shannon}$ = 0.0234). However, the α-diversity also increased with vehicle after seven days compared to baseline. This might result from a moderate direct antibacterial activity of polyethylenglycol (PEG), a constituent of the vehicle, which has previously been described for *S. aureus* [40]. However, it should be noted that treatment with ATx201 OINTMENT showed superior antibacterial effects compared to vehicle in all our studies. The β-diversity is a measure of dissimilarity in the taxonomic composition between samples which was analyzed by generalized UniFrac [41]. The change in microbiome community composition at day 7 compared to baseline was significantly greater for the ATx201 OINTMENT 2% twice-daily treatment group compared to vehicle (Figure 4A; Wilcoxon test, P = 0.0234). Moreover, the compositional plot displays that ATx201 OINTMENT 2% twice-daily significantly decreased the abundance of *S. aureus*, which is in alignment to the culture data obtained in this study considering the direction of the change. It further appears that other genera increase in abundance at the expense of *S. aureus* (Figure S4).

Overall, these data highlight that treatment of AD lesions with ATx201 reduces the abundance of *S. aureus* while increasing the skin Shannon diversity significantly compared to vehicle.

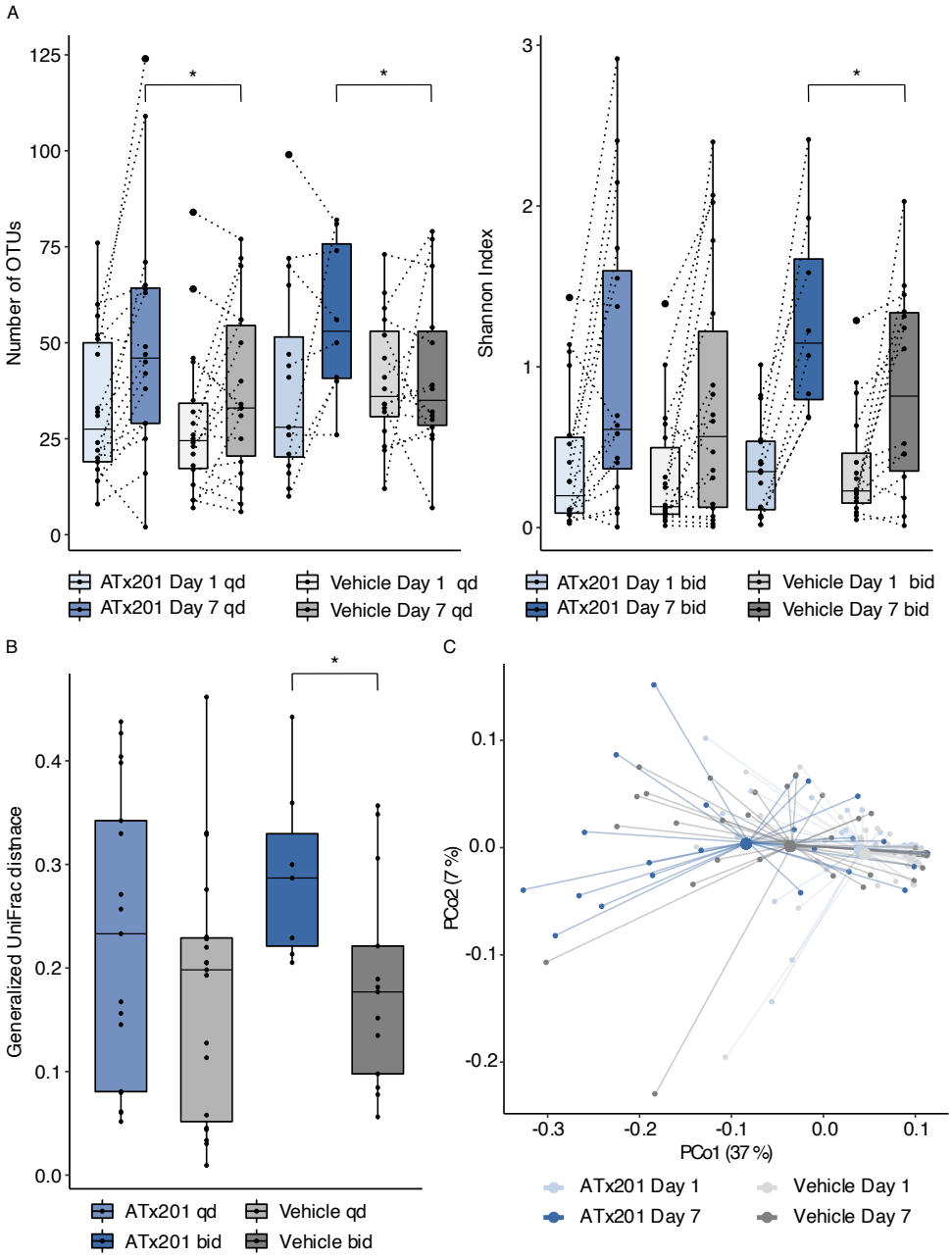

Figure 4. Effect of ATx201 OINTMENT 2% on the skin microbiome diversity compared to vehicle.

A Boxplot of the number of Operational Taxonomic Units (OTUs) and Shannon index per dosage group and visit. α-diversity was measured by counting the number of OTUs and calculating the Shannon Index. * = $p < 0.05$, Wilcoxon test. **B** Boxplot of the generalized UniFrac distance as measurement for β-diversity between day 1 and day 7 samples within a subject. * = $p < 0.05$, Wilcoxon test. **C** PCoA plots of samples by treatment group and time of sampling using generalized UniFrac distances calculated from OTU abundances. Paired samples are connected with a dotted line. Bid: twice-daily; qd: once-daily.

Discussion and Conclusion

In this study we describe ATx201 as a potent compound against *S. aureus*, including drug resistant strains *in vitro* and in a murine skin infection model. We find that ATx201 inhibits *S. aureus* by dissipating the proton motive force through its protonophore activity leading to a rapid shut down of bacterial biosynthesis and subsequently bacterial growth in a pH dependent manner. Targeting physical properties of the bacterial membrane has been described to reduce the likelihood of resistance evolution and benefit the elimination of persistent colonization [42]. Consistent with this we find that ATx201 has undetectable *de novo* resistance evolution strongly differentiating ATx201 from current cutaneous agents.

We showed that AD patients receiving ATx201 OINTMENT 2% twice-daily had a significant reduction in the abundance of *S. aureus* while increasing Shannon diversity of skin microbiome compared to vehicle after seven days. These findings are in alignment with the hypothesis that *S. aureus* overgrows the AD skin at the expense of the commensal microbiota leading to an inverse relationship between *S. aureus* abundance and microbiome diversity [7]. Accordingly, we expect that the observed increase in microbiome diversity following treatment with ATx201 compared to vehicle results from its potent activity against *S. aureus*, effectively limiting the proliferation of this dominant bacterium enabling other commensal microbes to regrow.

In order to further study the impact of ATx201 on the skin microbiota higher resolution metagenomics and longer treatment duration would be needed along with approaches to quantitate whether increases in Shannon diversity result primarily from a reduction in *S. aureus* or a regrowth of the commensal microbiota. Reduced *S. aureus* abundance and increased microbiome diversity have been linked to improved AD disease states [7]. In our study, ATx201 qualitatively improved AD lesions during 7 days of twice-daily treatment. However, we expect an extended treatment regimen would be required to further improve AD symptoms. Interestingly, AD patients are a reservoir for MRSA/*S. aureus* [4, 15, 43–45] and ATx201 could also be used to reduce *S. aureus*

in such patients to diminish the risk of transmitting pathogens to the public as well as contracting serious infections [46–48].

While complete removal of *S. aureus* is preferable with a newly developed antibiotic, we consider that ATx201 provides a very differentiated profile to conventional antibiotics, that typically are less selective. In contrast to fusidic acid, which has been demonstrated to select for resistant *S. aureus* during treatment AD patients [21], ATx201 does not seem to select readily for resistance in clinical use. Accordingly, ATx201 might be particularly well suited for longer term application in AD patients. Yet, the patient groups targeted by ATx201 could be extended beyond AD, to include decolonization of *S. aureus* to diminish the risk for patients on dialysis to develop catheter-related infections or prevent infectious complications post-surgery.

Interestingly, ATx201 achieves similar outcomes in AD patients as is expected from several treatments using live bacterial preparations. However, in contrast to such microbial therapies ATx201 would likely offer more effective manufacturing and reduce safety concerns related to bacterial products or donor derived materials. In conclusion, the data presented in this manuscript suggest that ATx201 could become a new treatment modality for AD.

Materials and Methods

Study Design

In this study, we tested ATx201, a small molecule, on its efficacy to reduce *S. aureus* colonization and propensity to evolve resistance *in vitro*. Various experimental cutaneous formulations of ATx201 were then tested in a superficial skin infection model and compared to current available antibacterial agents. Finally, a Phase 2 randomized, double-blind and vehicle-controlled trial was performed to investigate the impact of ATx201 OINTMENT 2% on *S. aureus* colonization and skin microbiome composition in patients with mild-to-severe atopic dermatitis. The clinical trial was conducted according to the provisions of the Declaration of Helsinki and its current

amendments, and the International Conference on Harmonization Guidelines for Good Clinical Practice. All studies comprising animals were conducted in accordance with the National Committee of Animal Ethics.

In vitro studies

Bacterial strains and reagents

The clinical strains methicillin- resistant *S. aureus* (MRSA) 43484 and 88779 (MRSA 14), were isolated from patients with skin abscesses and were obtained from UNION Therapeutics A/S. The laboratory strains of *S. aureus* used in this study (ATCC 29213, ATCC 25293, NCTC 8325, and JE2) were obtained from the Department of Veterinary Disease Biology at the University of Copenhagen. *S. aureus* RN4220 and its mutator derivative RN4220 Δ*mutS* were kindly provided by A. J. O'Neill (University of Leeds, UK).

Furthermore, remaining isolates included in this study were obtained from UNION Therapeutics A/S bacterial collection (Table S4).

All strains were cryopreserved in Brain Heart Infusion (BHI) broth supplemented with glycerol 15% (v/v) at –80°C and cultivated aerobically at 37°C in nutrient agar base no. 2 supplemented with calf blood 5% (v/v) (Oxoid, Basingstoke, UK). All bacterial cultures were grown in Mueller-Hinton broth (MHB) (Oxoid, Basingstoke, UK). When appropriate, culture medium was supplemented with ATx201 (Niclosamide; CAS: 50-65-7), rifampicin, fusidic acid, mupirocin, and retapamulin (Sigma-Aldrich) and the pH adjusted to different values with NaOH and HCl solutions of different concentrations (0.1 M, 1 M, 2 M and 5M).

Susceptibility analysis of ATx201 and comparators

Minimal inhibitory concentrations (MICs) were determined in triplicate by the broth microdilution method according to the recommendations of The Clinical & Laboratory Standards Institute (CLSI) [49].

Time-kill kinetics of ATx201

For the time-kill kinetics of ATx201, overnight cultures of strains MRSA 43484 and RN4220 were diluted in MHB to an OD_{600} = 0.1 and ~1.25×106 cells from these starter cultures were inoculated into flasks containing 25 mL of MHB (~5×104 CFU/mL) with different concentrations of ATx201 (drug-free, 1× MIC, 1.5× MIC and 4× MIC). The flasks were then incubated at 37°C and CFU counts were performed at time points 0, 24, 48, and 72 hours by spotting three 10 µL-drops of the diluted cultures on blood agar (BA) plates. All BA plates were incubated at 37°C and colonies were counted 24 hours later. Data were obtained in two independent experiments.

Bacterial growth inhibition study

MHB overnight culture of ATCC 29213 was sub-cultured in fresh broth and grown up to OD_{600} = 0.3. Afterwards, freshly grown cells were distributed to separate tubes and treated with 1×, 4× or 10× MIC of ciprofloxacin, rifampicin, erythromycin, vancomycin and ATx201. Untreated control was also included. Cells were further grown at 37°C and optical density was recorded at one-hour intervals at 600 nm and plotted against time.

Molecule biosynthesis rate determination assay

Macromolecule biosynthesis experiments were performed as described by Ling et al [50]. Briefly, *S. aureus* ATCC 29213 overnight culture was sub-cultured at 1:100 in minimal media (0.02 M HEPES, 0.002 M MgSO4, 0.0001 M CaCl2, 0.4% succinic acid, 0.043 M NaCl2, 0.5% (NH4)2SO4) supplemented with 5% tryptic soy broth (TSB) and grown to OD_{600} = 0.2. Cells were pelleted down by centrifugation and resuspended in fresh media followed by incubation for 15 minutes (for cell wall synthesis 30 minutes) with negative control DMSO, ATx201, CCCP or

positive control of DNA, RNA, protein or cell wall synthesis inhibitors ciprofloxacin, rifampicin, erythromycin and vancomycin, respectively.

The MICs of *S. aureus* ATCC 29213 for ciprofloxacin, rifampicin, erythromycin and vancomycin were found to be 0.25, 0.003, 0.5 and 1 μg/mL, respectively. After incubation, cells were pulse labelled individually by radiolabeled DNA, RNA, protein and cell wall synthesis precursors (50 μCi) 3H-thymidine, (5 μCi) 3H-uridine, (50 μCi) 3H-leucine and (5 μCi) 3H-glucosamine hydrochloride per mL, respectively, for 20 minutes (for cell wall synthesis cells 10 minutes). Finally, samples were precipitated with equal volume of cold 30% TCA. Precipitates were membrane filtered and subsequently washed twice with cold 15% TCA and twice with water. Filters were air dried and stored in 10 mL scintillation vials. Finally, 3 mL scintillation fluid was added to each vial and a 3H count was taken in a Beckman Coulter LS6500 liquid scintillation counter for 1 minute. Radioactive count of control (DMSO) treated sample was considered as 100% precursor incorporation in macromolecule synthesis. Accordingly, percentage rate of other treated samples was calculated. Data were obtained in two independent experiments.

DiSC3(5) fluorescence-based membrane potential study

MHB overnight cultures of ATCC 29213 and MRSA 43484 were sub-cultured 1:100 in fresh MHB media and grown to OD_{600} = 0.2. Grown cells were diluted to OD_{600} = 0.1 in MHB and subsequently labelled with 1μM 3, 3′-dipropylthiadicarbocyanine iodide [DiSC3(5)]. The fluorescence spectra of labelled cells were taken in a microplate reader (Synergy H1, Holm & Halby) at Ex/Em wavelengths 622nm/670nm over time. After reading of initial stable DiSC3(5) spectra, labelled cells were treated with DMSO, ATx201, CCCP, Vancomycin, Ciprofloxacin, Rifampicin or Erythromycin and change of DiSC3(5) fluorescence was recorded over time.

Cytoplasmic pH change measurement

MHB overnight culture of ATCC 29213 was sub-cultured 1:100 in fresh media and grown to OD_{600} = 0.2 and subsequently incubated with 10 µg/mL of BCECF-AM, a pH sensitive cytoplasmic specific dye, for 30 minutes at 30°C. Incubated cells were pelleted down by centrifugation and resuspended in phosphate buffer saline (PBS: 1.37 M NaCl, 27 mM KCl, 100 mM Na2HPO4, 18 mM KH2PO4, pH 7.4) supplemented with 25 mM glucose. Subsequently, the cells were adjusted to OD_{600} = 0.2. The cell suspension was kept at room temperature for 1 hour to equilibrate the cytoplasmic pH. BCECF fluorescence of labelled cells was recorded in a PerkinElmer LS50B fluorescence spectrometer at Ex/Em wavelengths 485 nm/520 nm using the time drive application of FLWINLAB software. After initial stable reading of BCECF spectra, labelled cells were treated with DMSO, ATx201, nigericin and CCCP individually. The changes of BCECF fluorescence upon treatments ($FU_{2.8\ minute} - FU_{0.2\ minute}$) were plotted. Data were obtained in two independent experiments.

Estimation of spontaneous mutation rates

A fluctuation analysis was adapted from Rosche et al. and Gerrish et al. [51, 52]. Approximately 2.5×104 cells from late-exponential phase cultures were inoculated into 250 mL of MHB (102 CFU/mL) and distributed into 24 tubes containing 10 mL of MHB with ~103 cells each. These were incubated at 37°C with agitation (125 rpm) and grown to late stationary phase. After incubation, ~109 cells were spread onto MHA plates containing 0.25, 0.375 and 1 µg/mL of ATx201 by centrifuging 1 mL of culture for 3 minutes at 5000 rpm, partially discarding the supernatant and resuspending the pellet in the remaining media. The rate of mutational resistance to rifampicin was determined as a control, whereas the rates of mutational resistance to fusidic acid, mupirocin and retapamulin were determined for comparison purposes (~108 cells). The number of resistant colonies was counted after 48 hours of incubation at 37°C. The average final

number of cells (Nf) was obtained from 4 random cultures by spotting triplicate 10 µL-drops of 1:10 serial dilutions in BA.

The most likely number of mutations per culture (m) that gave rise to the distribution of mutants observed in the parallel cultures was estimated with a maximum likelihood method using the recursive formula implemented by Gerrish et al. [52]. The mutation rate (µ) (number of mutations per cell per division) was then calculated by dividing m by Nf. The mutation rate estimates represent the mean value of two independent experiments. The frequency of mutants (F) was obtained by multiplying the average number of colonies from the 24 cultures by the volume factor and dividing the resulting value by the Nf. Strains RN4220 and RN4220 ΔmutS were used to validate the fluctuation assay.

Susceptibility testing after 40-day passage

The *in vitro* development of resistance to ATx201 were carried out by subjecting MRSA 16115 (veterinary isolate) and 38127 (clinical isolate) in liquid broths to serial passages to sub-inhibitory concentrations of ATx201 (1/4 MIC) for 40 passages. After every 10 passages, the MIC of ATx201 was determined. The MICs were determined by microbroth dilution according to CLSI guidelines [49]. *S. aureus* ATCC 29213 was included for quality control purposes.

Mouse studies

Bacterial strains and mice

Strains MRSA 43484, MRSA 88779 and MRSA 86446, obtained from Statens Serum Institute, Copenhagen, Denmark and representing different resistance and genomic backgrounds, were selected to inoculate the mice. Bacterial suspensions for inoculation of mice were prepared from overnight cultures on 5% blood agar plates immediately before inoculation by suspending colonies in sterile 0.9% saline to 9 $\log_{10}$ CFU/mL.

Female BALB/c mice, 10–12 weeks old (Taconic, Ejby, Denmark) were housed at Statens Serum Institut. All animal experiments were approved by the National Committee of Animal Ethics, Denmark. License No. 2013-15-2935-00900.

Experimental skin wound infection model

The experiments were conducted essentially as described by Vingsbo et al. [34]. Female BALB/c mice, 10–12 weeks old (Taconic, Ejby, Denmark), were anaesthetized and shaved on a 2–3 cm^2 skin area of the lower back. A disposable 7 mm dermal curette (Integra, York, PA, USA) was used to induce a minor superficial skin wound on a 1 cm^2 area, which was inoculated with ~7 log^{10} CFU of bacteria. Four days after inoculation and treatment, the mice were sacrificed and the entire infected skin area with underlying tissue was cut out and homogenized in 1 mL of saline using gentleMACS™ C-tubes (Miltenyi Biotec, Lund, Sweden) on a Dispomix® Drive (Medic Tools AG, Zug, Switzerland) running at 4000 rpm for 10 seconds. Tissue homogenates were serially diluted 10-fold in saline containing 0.1% Triton X-100 (T-8787; Sigma–Aldrich Inc., St Louis, MO, USA). Then, 20 µL spots were applied in duplicate to NaCl agar supplemented with polymyxin B (SSI Diagnostica). In addition, 100 µL of the 10^{-2} dilution was spread on a separate plate to increase sensitivity and minimize the risk of a false negative due to carry-over of high drug concentrations from the wound. Plates were incubated at 35°C in ambient air for 20–48 hours.

Formulation preparation of ATx201 for animal studies

The ATx201 formulations used for the skin wound infection model were prepared at UNION Therapeutics A/S. The comparators used were a Fucidin® and Bactroban®. Fucidin® is a commercial product registered in Denmark: (Fusidic acid 2%; LEO Pharma A/S, Ballerup, Denmark). Bactroban® is the commercial product registered in Denmark (Bactroban® nasal 2%, GSK).

Treatment of skin infection

Mice were treated twice-daily at approximately 24, 30, 48, 54, 72 and 78 hours after inoculation by applying topically 0.05 mL of the ATx201 formulation or the comparators to the lesion area. Skin samples were collected the day after completed treatment for CFU determination. Carry-over effects were minimized by performing the CFU determinations >18 hours after the last treatment and homogenizing, diluting and plating the samples immediately.

Phase 2 study design

This Phase 2, prospective, randomized, double blind, placebo-controlled single-center trial was designed to evaluate the safety and efficacy of ATx201 OINTMENT 2% (drug substance: niclosamide) as a cutaneous decolonizing agent in AD patients versus vehicle (DECOLAD Part 2, NCT03009734). The study was performed from December 2016 until March 2018 in the Department of Clinical Pharmacology at the Medical University of Vienna and was conducted according to the provisions of the Declaration of Helsinki and its current amendments, and the International Conference on Harmonization Guidelines for Good Clinical Practice (formal ethical approval for the clinical trial can be found at the end of the chapter).

For each patient two treatment areas were selected for treatment (ATx201 OINTMENT 2% or vehicle), and patients were randomized to receive active left and vehicle right or vice versa. A third (untreated) lesion was selected. An additional grouping factor was once-daily (qb) versus twice-daily application (bid). If possible, target lesions were to be chosen symmetrically on the left and on the right side of the body, respectively. However, if there were no target lesions on both sides of the body, the investigator could choose two target lesions on the same side of the body.

The primary objective of the study was to demonstrate the safety and tolerability of ATx201 OINTMENT 2% in patients with AD, and to assess efficacy of ATx201 OINTMENT 2% in eradicating *S. aureus* compared to vehicle after 7 days of treatment. Treatment success as primary

efficacy criteria was defined as a 100-fold reduction in the *S. aureus* CFU/cm^2 of skin lesions after 7 days of treatment. The secondary efficacy criterium was defined as the relative decrease of *S. aureus* CFU/cm^2 on treated skin after 7 days treatment of the ATx201 OINTMENT 2% group, compared to vehicle and untreated area.

The secondary objective of the study was to assess the impact of ATx201 OINTMENT 2% on the treated AD lesion. AD lesions were assessed using the IGA score, EASI score, and VAS pruritus score.

The study was composed of male and female subjects between 18 and 70 years of age with signed and dated informed consent obtained and diagnosed with AD (according to the Hanifin & Rajka criteria [53] presenting as localized disease with three lesions. Further, patients with two individual lesions each covering an area between 10-200 cm^2, having an investigator's global assessment (IGA) score between 1-4 and colonized by *S. aureus* with at least 1,000 CFU/cm^2, and a third localized lesion of area between 10-200 cm^2 with IGA score between 1-4 were included. The full list of inclusion and exclusion criteria is provided in Table S5.

The study consisted of a screening visit, a 7-day treatment period, and an end-of-study visit on day 14. Patients with AD and colonized by *S. aureus* were treated once or twice-daily on two defined treatment areas between 10-200 cm^2 using either ATx201 OINTMENT 2% or matching vehicle (split-body design). The treatments were blinded to both patients and physicians. ATx201 OINTMENT 2% had to be dosed with a thin layer covering the entire lesion as instructed by the clinical personnel at the randomization visit. Only the first treatment application was performed by the investigator, the remaining applications were done by the patients.

Sample collection

Skin swabs were obtained at screening visit (up to 5 lesions), Baseline (day 1) and end-of-treatment (EOT) (day 7) for quantitative culture of *S. aureus* using a sterile cotton swab dipped into a buffer of 0.1% Triton X-100 in 0.075 M phosphate buffer, pH 7.9. A template of 6.4 cm^2

was streaked with a cotton swab for 10 seconds and solubilized in 1 mL buffer. This sample collection procedure has been shown to maximize the recovery of *S. aureus* from skin of AD patients [54].

Quantitative culture of *S. aureus*

All collected liquid samples were submitted and processed immediately. The swap buffer solution was vortexed mildly to release bacteria. Samples of the skin swab solution were serially diluted and streaked on ChromID agar plates. After incubation at 37°C over 24 hours the *S. aureus* specific colonies were counted, and the amount of bacterial load was described as CFU per cm^2 of the investigated AD skin area. The cut-off value for definition of colonization of lesions with S. aureus was defined as 1000 CFU/cm^2.

Quantification of skin microbiome diversity

Skin samples obtained at Baseline and EOT were further used for quantification of skin microbiome diversity (α- and β-diversity) by 16S rDNA sequencing using an established methodology [7]. Briefly, DNA was extracted from skin swaps using NucleoSpin® 96 Soil (Macherey-Nagel). rRNA genes (16S) were amplified from purified genomic DNA using the primers described on Table 5, with Ilumina adapter attached. Sequencing was done on an Illumina MiSeq desktop sequencer using the MiSeq Reagent Kit V3 (Illumina) for 2x 300 bp paired-end sequencing. The 64-bit version of USEARCH 10.0 [55], mothur 1.38 [56], and in-house scripts were used for bioinformatics analysis of the sequence data. Taxonomic assignment of OTUs was done using SINTAX with a cutoff value of 0.8 [57] against RDP training set v16 [58]. All analyses, except for calculation of Shannon index, were done on rarefied data.

Table 5. Selected primers.

515F(5'**CGTCGGCAGCGTCAGATGTGTATAAGAGACAG**GTGYCAGCMGCCGCGGTAA -3')
806R (5'**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**GGACTACNVGGGTWTCTAAT-3')

The α-diversity of the samples was assessed by counting the number of OTUs (operational taxonomical units, similar to species richness) and calculating the Shannon index. The Shannon index does not only take the number of OTUs of a community into account, but also the relative abundance of the OTUs. It will give a low diversity score to a community dominated by a few species (even though the total species number may be high), while communities where many different species have similar abundances will receive a high diversity score.

Whereas to measure the β-diversity, we used generalized UniFrac [41], which is an intermediate between weighted and unweighted UniFrac ($\alpha = 0.5$). The β-diversity is a measure of dissimilarity in the taxonomic composition between samples. One measure of β-diversity is UniFrac. The UniFrac distance takes taxonomic distance of the OTUs into account: two samples consisting of different species from the same genus will have a shorter UniFrac distance between them than two samples consisting of species from different phyla. The UniFrac distances can also take abundance into account. If full weight is given to the abundances ($\alpha = 1$, where α determines the weight given to abundance differences) it is called a weighted UniFrac. In such case, the UniFrac distance may be high between two samples that contain the exact same species in very different abundances. A UniFrac distance that does not take abundance into account ($\alpha = 0$), working only with presence-absence data, is called an unweighted UniFrac.

Statistical analysis

In vivo model

CFU data was $\log_{10}$ transformed prior to analysis. Samples that were CFU negative (i.e. below the lower detection limit of 50 CFU) were assigned a CFU value of 50. ANOVA and Dunnett's multiple comparison test were used to compare log10 CFU values for multiple groups. P-values < 0.05 were considered significant. All statistical comparison was made using the software GraphPad Prism 6.00 for Windows, GraphPad Software, CA USA.

Clinical study

Statistical tests regarding the primary endpoint of the study were performed using a Chi-Square test for dependent samples in SAS (Test of Symmetry according to Bowker). To keep a global alpha of 0.05 (two-sided), each of the two hypotheses were tested at a significance level of alpha = 0.025, two-sided.

Statistical test for change in *S. aureus* abundance level (CFU) was performed using Wilcoxon Test. Statistical tests regarding the microbiome analysis were performed using Wilcoxon Test. Notwithstanding statistical tests are defined in the respective result section.

All statistical tests were made using R (version 3.3 or greater).

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Author contributions: R.T.K. and M.O.A.S. conceived and secured funding for the studies. E.D., A.W., C. M., D.S., P.L., J.P., T.P.S., B.J., C.F. and L.G. performed the microbiological experiments. H.L., C.B., M.B. S.E., A.N.P., Z.A.Ö., P.M., T.Q., M.W., G.S., E.G. and M.Z. were involved in the planning and execution of the clinical trial, bacterial quantification and interpretation of study results. J.H., C.L. performed the mouse studies and interpreted the *in vivo* data. P.L., H.N., T.R., P.P. worked on the formulation development. H.K.J., H.W., F.M. and P.B. provided microbiological isolates and interpreted the microbiological data. N.S. and H.B.N.

performed and interpreted the microbiome experiments. W.K., E.M. provided input to the microbiological experiments. A.W., C.M. and M.O.A.S wrote the manuscript. All authors reviewed the manuscript.

Competing interests: E.D., A.W., C. M., D.S., P.L. and J.P. were all employees of UNION Therapeutics A/S at the time these studies were performed. R.T.K and M.O.A.S. are shareholders of UNION Therapeutics A/S. UNION Therapeutics A/S owns patents around ATx201. E.G. and G.S. are advisors for UNION therapeutics.

Data and materials availability: All data associated with this study are present in the paper or in the Supplementary Materials.

Supplementary Material

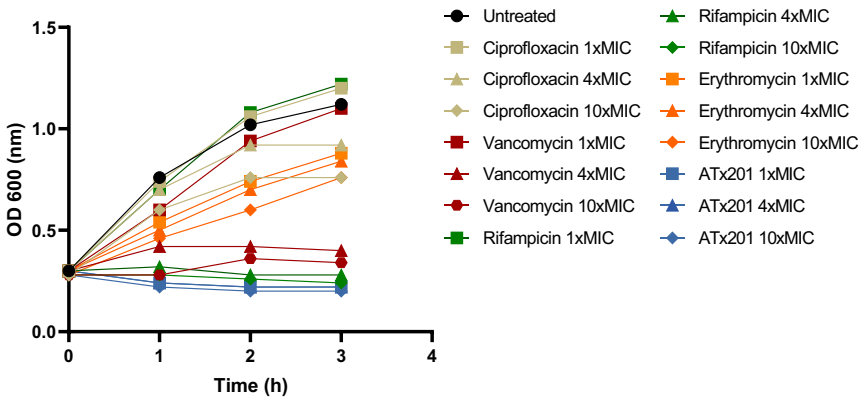

Figure S1. 1×MIC of standard DNA, RNA, protein and cell wall synthesis inhibitor do not arrest growth of exponentially grown ATCC 29213. Cells were grown to 0.3 OD at 600 nm then distributed to separate tubes and incubated with 1× and 4× and 10× MIC of ciprofloxacin, rifampicin, erythromycin, vancomycin or ATx201 for another 3 hours and cell OD was recorded after every hour and plotted in growth curve. In the right panel, MIC fold of antimicrobial concentrations are listed, which follow the same order as growth curve lines from top to bottom. 1×MIC of ciprofloxacin, rifampicin, erythromycin, vancomycin and ATx201 are 0.25, 0.003, 0.5, 2 and 0.25 µg/mL, respectively.

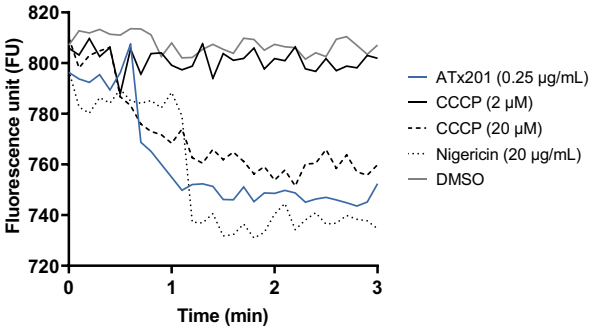

Figure S2. ATx201 acts as a proton carrier. Effect of ATx201, nigericin, CCCP or DMSO treatments on BCECF fluorescence in ATCC 29213 are plotted as function of time.

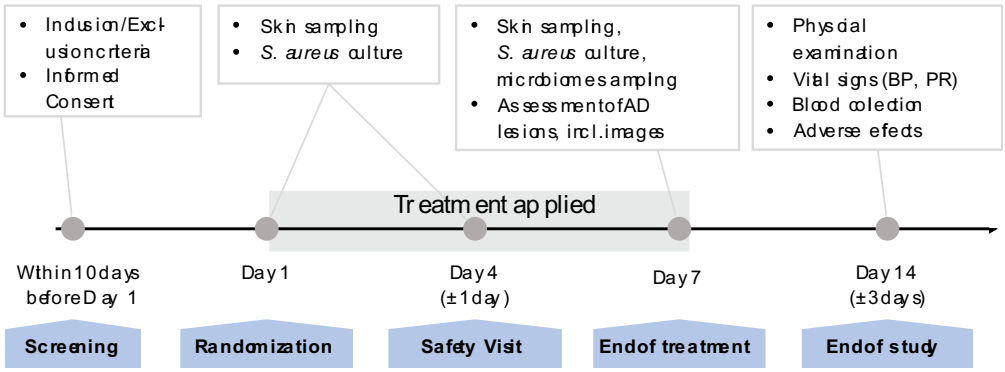

Figure S3. Study design of the placebo-controlled, double-blind, split-body-design Phase 2B study.

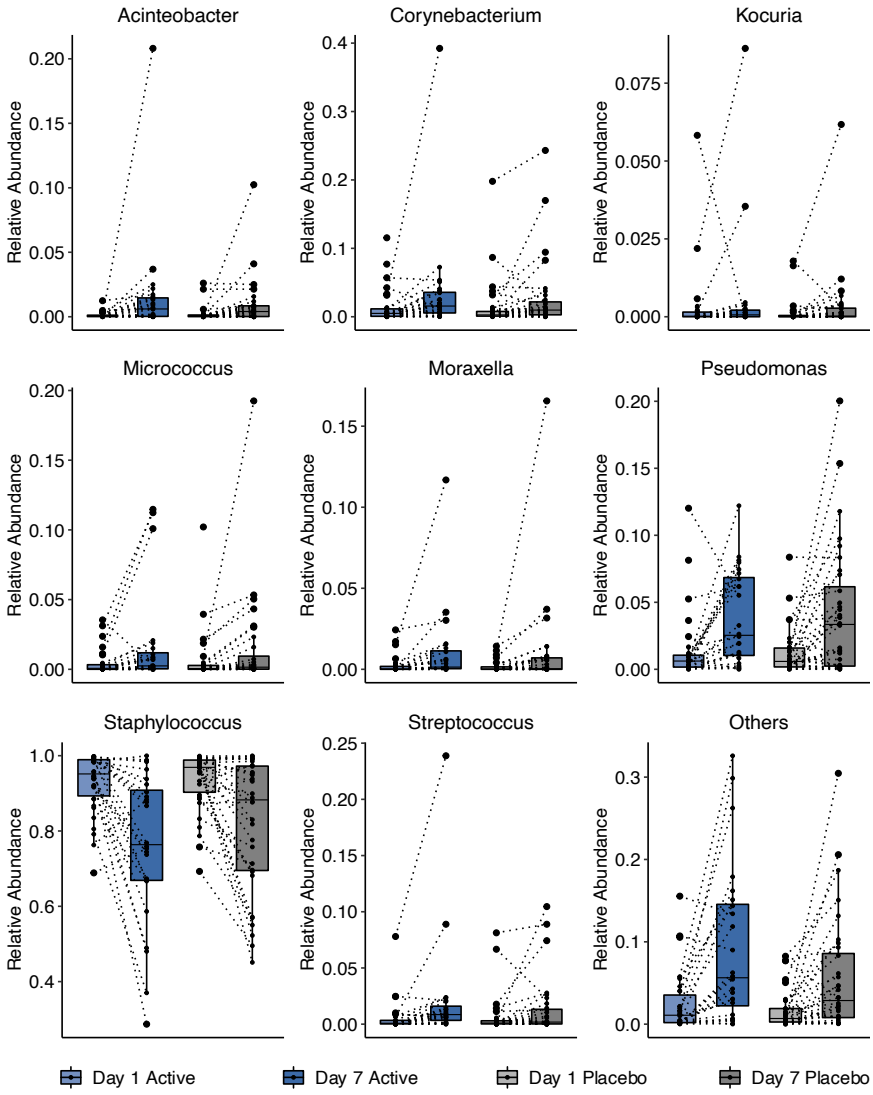

Figure S4. Genus-level relative abundances per treatment regimen. Dotted line visualizes each paired sample of the same subject.

Table S1. *In vitro* susceptibility of *S. aureus* clinical isolates and *S. aureus* ATCC 29213 reference strain. Values above clinical breakpoint are highlighted in red. ND: not determined

	MIC (µg/mL)					
	ATx201	**Fusidic acid**	**Mupirocin**	**Retapamulin**	**Clindamycin**	**Vancomycin**
ATCC 29213	0.5	0.06	0.125	0.03	0.125	1
Newman	0.25	0.25	0.25	0.03	0.125	2
MRSA 01	0.25	0.125	0.25	0.06	0.125	1
MRSA 02	0.25	0.5	0.25	0.06	0.25	2
MRSA 03	0.25	16	0.25	0.03	0.125	1
MRSA 04	0.25	0.25	0.25	0.06	0.125	2
MRSA 05	0.25	0.125	0.125	0.03	0.125	1
MRSA 06	0.25	0.125	0.25	0.03	0.125	2
MRSA 07	0.125	0.25	0.25	0.06	0.125	1
MRSA 08	0.25	0.125	> 16	0.03	> 16	1
MRSA 09	0.125	0.25	0.25	0.06	0.25	2
MRSA 10	0.25	1	0.25	0.06	0.25	1
MRSA 11	0.25	1	0.5	0.06	> 16	1
EEFIC 01	0.25	4	0.125	0.03	0.125	1
EEFIC 02	0.25	4	0.125	0.03	0.125	1
MRSA 12	0.5	4	0.125	0.03	ND	ND
MRSA 13	0.25	4	0.25	0.06	0.125	1
MSSA 01	0.5	4	0.125	0.03	0.125	1
MSSA 02	0.25	4	0.125	0.03	0.125	1
MRSA 14	0.25	> 16	0.125	0.03	> 16	1
MRSA 15	0.25	> 16	0.25	0.06	0.125	2
MRSA 16	0.25	0.25	> 16	0.06	0.25	1
MRSA 17	0.25	4	> 16	0.03	> 16	2
MRSA 18	0.25	0.25	0.125	0.06	0.125	1
MRSA 19	0.25	0.125	0.25	0.03	0.125	1
MRSA 20	0.25	0.125	0.25	0.06	0.06	1
MRSA 21	0.25	0.125	0.125	0.125	0.03	1
MRSA 22	0.25	> 16	0.25	0.06	0.06	0.5
MRSA 23	0.25	0.25	0.25	0.06	0.125	1
MRSA 24	0.25	0.25	0.25	0.06	0.125	2
MRSA 25	0.25	16	0.25	0.06	0.125	1
MRSA 26	0.5	0.5	0.5	0.06	> 16	1
MRSA 27	0.5	0.25	0.25	0.06	0.125	1
MRSA 28	0.25	0.06	0.5	0.03	0.06	1
MRSA 29	0.5	4	0.25	0.06	0.125	1
MRSA 30	0.25	0.125	0.125	0.03	0.06	1
MRSA 31	0.25	0.25	0.25	16	> 16	1
MRSA 32	0.25	0.5	0.25	0.06	0.125	1
MRSA 33	0.25	0.5	0.25	0.06	0.125	1
MRSA 34	0.25	0.25	0.25	0.06	0.125	1
MRSA 35	0.25	16	0.5	0.03	> 16	1
MRSA 36	0.25	8	0.25	0.06	0.06	1
MRSA 37	0.25	0.5	0.25	0.06	0.125	1
MRSA 38	0.25	8	0.125	0.06	0.125	1
MRSA 39	0.25	8	0.25	0.06	0.06	1
MRSA 40	0.25	0.125	0.25	0.03	0.06	1
MRSA 41	0.25	> 16	0.125	0.02	0.06	1
MRSA 42	0.25	0.25	0.25	0.06	> 16	1

	MIC (µg/mL)					
	ATx201	**Fusidic acid**	**Mupirocin**	**Retapamulin**	**Clindamycin**	**Vancomycin**
MRSA 43	0.25	0.125	0.25	0.06	0.06	1
MRSA 44	0.25	4	0.25	0.02	0.03	1
MRSA 45	0.25	0.06	0.13	0.02	0.06	1
MRSA 46	0.25	0.06	0.13	0.02	0.03	1
MRSA 47	0.25	0.06	0.25	0.02	> 16	0.5
MRSA 48	0.25	2	0.13	0.02	0.03	0.5
MRSA 49	0.25	0.25	0.25	0.02	0.03	2
MRSA 50	0.25	0.03	0.25	0.02	0.02	1
MRSA 51	0.125	0.13	0.25	0.03	0.06	1
MRSA 52	0.25	0.06	0.25	0.02	0.03	1
MRSA 53	0.25	0.25	0.25	0.03	0.06	1
MRSA 54	0.25	0.125	0.25	0.03	0.06	2
MRSA 55	0.25	4	0.25	0.02	0.03	0.5
MRSA 56	0.25	0.06	0.25	< 0.01	0.03	1
MRSA 57	0.25	0.125	0.125	0.02	0.03	1
MRSA 58	0.25	4	0.125	0.02	0.06	1
MRSA 59	0.25	0.06	0.125	0.02	0.03	1
MRSA 60	0.25	8	0.25	0.02	0.06	0.5
MRSA 61	0.25	0.06	0.25	0.02	0.03	2
MRSA 62	0.25	0.06	0.25	0.02	0.06	2
MRSA 63	0.25	0.125	0.125	0.02	0.06	1
MRSA 64	0.25	0.25	0.25	0.02	0.06	1
MRSA 65	0.25	0.06	0.25	0.02	0.03	1
MRSA 66	0.25	0.06	0.25	0.02	0.03	1
MRSA 67	0.25	0.25	0.125	0.02	0.03	1
MRSA 68	0.25	0.125	0.25	0.02	0.03	1
MRSA 69	0.25	4	0.125	< 0.01	0.03	1
MRSA 70	0.125	8	0.25	0.02	0.06	1
MRSA 71	0.25	0.06	0.125	< 0.01	0.02	1
MRSA 72	0.25	16	0.25	0.03	0.06	1
MRSA 73	0.5	0.25	0.25	0.03	0.125	1
MRSA 74	0.25	0.25	0.25	0.03	0.125	2
MRSA 75	0.5	0.5	0.5	0.03	0.125	1
MRSA 76	0.25	0.25	0.25	0.03	0.125	1
MRSA 77	0.25	16	0.25	0.03	0.125	2
MRSA 78	0.5	0.125	0.25	0.03	0.06	1
MRSA 79	0.25	0.5	0.25	< 0.01	0.03	1
MRSA 80	0.5	0.125	0.25	0.02	0.06	1
MRSA 81	0.5	0.25	0.25	0.03	0.125	1
MRSA 82	0.25	8	0.25	0.03	0.125	2
MRSA 83	0.5	0.06	0.25	0.03	0.06	1
MRSA 27b	0.25	8	0.25	0.02	0.06	1
MRSA 84	0.25	8	0.25	0.03	0.125	2
MRSA 85	0.5	4	0.25	0.03	0.125	1
MRSA 86	0.25	> 16	0.25	0.03	0.06	2
MRSA 87	0.25	0.125	0.25	0.02	0.06	1
MRSA 88	0.25	0.06	0.25	0.03	0.06	1
MRSA 89	0.5	0.06	0.25	0.02	> 16	1
MRSA 90	0.5	16	0.25	0.06	0.125	1
MRSA 91	0.25	0.25	0.25	0.06	0.125	1
MRSA 92	0.25	8	0.25	0.06	0.06	1

	MIC (μg/mL)					
	ATx201	**Fusidic acid**	**Mupirocin**	**Retapamulin**	**Clindamycin**	**Vancomycin**
MRSA 93	0.25	0.02	0.25	0.02	0.03	2
MRSA 94	0.25	0.125	0.25	0.03	0.125	2
MRSA 95	0.25	8	0.25	0.03	0.125	2
MRSA 96	0.25	0.125	0.125	0.03	> 16	1
MRSA 97	0.25	8	0.25	0.03	0.06	1
MRSA 98	0.5	0.06	0.5	0.03	> 16	1
MRSA 99	0.25	0.125	0.5	0.03	0.125	1
MRSA 100	0.25	0.125	0.5	1	0.25	1
MRSA 101	0.5	8	0.25	0.03	0.06	1
MRSA 102	0.25	0.06	0.25	0.03	0.06	2
MRSA 103	0.25	8	0.25	0.03	0.125	1
MRSA 104	0.5	0.25	0.25	0.03	0.125	1
MRSA 105	0.25	0.125	0.25	0.03	0.125	1
MRSA 106	0.25	0.125	0.25	0.06	0.125	1
MRSA 107	0.25	0.25	0.25	0.06	0.125	2
MRSA 108	0.25	4	0.25	0.03	0.125	1
MRSA 109	0.25	0.25	0.25	0.03	0.125	1
MRSA 110	0.25	0.06	0.125	0.03	0.125	1
MRSA 111	0.5	8	0.25	0.03	0.125	1
MRSA 112	0.25	0.06	0.25	0.03	0.06	1
MRSA 113	0.25	0.125	0.25	0.03	0.06	1
K000796	0.25	8	0.5	0.03	0.125	1
K115688	0.25	0.125	0.25	0.03	0.125	2
K000866	0.25	8	0.5	0.03	0.125	1
K000864	0.25	0.25	0.25	0.03	0.125	1
K000863	0.25	1	0.5	0.03	0.125	1
K115689	0.25	0.125	0.25	0.03	0.125	2
K000772	0.25	> 16	0.125	0.06	0.125	1
K115498	0.25	0.125	0.5	0.03	0.125	2
R000024	0.25	16	0.5	0.06	0.125	1
R000020	0.5	0.125	0.5	0.03	0.125	1
R000019	0.5	0.125	0.5	0.06	0.125	2
U115579	0.25	0.25	0.5	0.06	0.125	1
U115370	0.25	0.5	0.125	0.06	0.125	1
U114660	0.25	0.25	0.25	0.06	0.125	1
D115584	0.25	0.25	0.5	0.06	0.125	2
E115740	0.5	0.25	0.25	0.06	0.125	1
E115810	0.25	0.25	0.25	0.06	0.125	1
T115628	0.25	8	0.25	0.03	0.06	2
T000274	0.25	0.5	0.5	0.06	0.125	1
T115691	0.5	0.25	0.25	0.03	0.125	1
T115903	0.5	8	0.5	0.03	0.125	1
T116122	0.25	0.125	0.5	0.03	0.125	1
T115015	0.5	0.25	0.5	0.06	0.125	2
C115273	0.5	0.25	0.25	0.03	0.125	1
C000040	0.5	0.25	0.25	0.03	0.125	1
C115690	0.25	8	0.125	0.25	0.5	2
C115561	0.25	0.125	0.5	0.03	0.125	1
C115445	0.5	0.25	0.25	0.03	0.125	1
C115263	0.25	0.125	0.25	0.03	0.06	2
C115303	0.5	0.25	0.25	0.03	> 16	1

	MIC (µg/mL)					
	ATx201	**Fusidic acid**	**Mupirocin**	**Retapamulin**	**Clindamycin**	**Vancomycin**
C115268	0.5	0.25	0.5	0.03	0.125	1
C115295	0.25	0.125	0.25	0.03	0.125	1
C115242	0.5	8	0.25	0.03	0.06	1
C115427	0.25	0.125	0.25	0.03	0.06	1
C000041	0.25	0.25	0.25	0.03	0.06	1
E5-1048654	0.25	0.5	0.25	0.06	0.125	1
9-2955245	0.25	0.25	0.25	0.06	0.125	1
E5-1046019	0.25	0.25	0.25	0.03	0.125	1
E5-1046020	0.5	0.25	0.25	0.06	0.125	1
E5-1047585	0.25	0.25	0.25	0.03	0.125	1
E5-1038294	0.5	0.25	0.25	0.03	0.125	1
E5-1035779	0.5	0.125	0.5	0.03	0.125	1
9-1862936	0.5	0.125	0.25	0.03	0.125	1
E5-1033091	0.5	0.03	0.25	0.02	0.06	1
9-26422166	0.5	8	0.25	0.03	0.125	1
9-2642158	0.25	0.25	0.25	0.06	0.125	1
E5-1035775	0.5	> 16	0.25	0.06	0.125	1
E5-1029558	0.25	16	0.5	0.03	0.125	1
E5-1038279	0.5	4	0.25	0.03	0.125	1
E5-1039697	0.25	0.5	0.25	0.06	0.125	1
E5-1041979	0.5	0.25	0.5	0.03	0.125	1
E5-1035284	0.25	0.25	0.25	0.03	0.125	1
E5-1030469	0.25	0.125	0.25	0.03	0.125	2
E5-1030472	0.5	0.25	0.5	0.06	0.125	1
E5-1041977	0.5	16	0.5	0.03	0.125	2
E5-1041987	0.5	16	0.25	0.03	0.125	1
E5-1039684	0.5	16	0.25	0.06	0.125	1
E5-1041980	0.25	0.25	0.25	0.03	0.125	1
E5-1033088	0.25	0.25	0.25	0.03	0.125	1
E5-1035277	0.5	16	0.5	0.03	0.125	1
E5-1046096	0.5	0.5	0.5	0.06	0.125	1
E5-1046085	0.5	8	0.5	0.06	0.125	2
9-2625962	0.5	0.25	0.5	0.03	0.125	1
E5-1043668	0.25	1	0.25	0.06	0.25	1
E5-1048428	0.25	0.5	0.25	0.06	0.25	1
E5-1047924	0.5	0.25	0.5	0.03	0.125	1
E5-1047606	0.5	8	0.5	0.03	0.125	1
E5-1046070	0.25	0.25	0.5	0.03	0.125	1
E5-1046298	0.25	0.125	0.25	0.03	0.125	1
E5-1046296	0.5	0.125	1	0.03	0.125	1
E5-1046297	0.5	0.125	0.25	0.06	0.125	1
E5-1043184	0.5	16	0.5	0.03	0.125	1
E5-1038286	0.25	0.25	0.5	0.06	0.125	1
E5-1037958	0.5	16	0.5	0.06	0.125	1
E5-1037971	0.25	0.25	0.25	0.03	0.125	1
E5-1033076	0.5	0.25	0.25	0.03	0.125	1
E5-1033076	0.5	0.25	0.25	0.03	0.125	1
E5-1029252	0.25	0.25	0.5	0.03	0.125	1
E5-1030440	0.25	0.25	0.25	0.06	0.06	1
E5-1030482	0.125	16	0.25	0.02	0.06	1
E5-1046074	0.25	0.125	0.25	0.06	0.125	1

	MIC (µg/mL)					
	ATx201	**Fusidic acid**	**Mupirocin**	**Retapamulin**	**Clindamycin**	**Vancomycin**
E5-1048204	0.25	0.25	0.25	0.03	0.06	2
E5-1048670	0.125	0.5	0.5	0.06	0.125	2
E5-1046039	0.25	0.25	0.25	0.06	0.125	1
E5-1045179	0.25	0.25	0.25	0.06	0.125	1
E5-1046723	0.25	0.5	0.25	0.06	0.125	2
	ATx201	**Rifampicin**				
RN422	0.25	0.004				

Table S2. Baseline demographics per regimen group.

	Once-daily (N = 18)	**Twice-daily (N = 18)**
Gender (N (% of total))		
Female	5 (27.8%)	13 (72.2%)
Male	13 (72.2%)	5 (27.8%)
Ethnicity (N (% of total))		
Asian	1 (5.6%)	2 (11.1%)
Caucasian	17 (94.4%)	16 (88.9%)
Age, yr (± SD)	31.56 (± 9.85)	34.17 (± 14.05)
Weight (kg)	69.32 (±12.01)	74.08 (± 18.03)
Height (cm)	173.78 (± 8.67)	170.44 (± 8.45)
BMI (kg/m^2)	22.86 (± 2.86)	25.34 (± 5.44)

Table S3. Summary of AEs by Organ class and detailed description of administration site conditions.

Summary of AEs by organ class per dosage group and relationship

SOC	qd	bid	Total	% of all AEs (51)	% of SAF (43)
Blood and lymphatic system disorders	**2**	**0**	**2**	3.9	9.1
Not Related	1	0	1		
Not likely	1	0	1		
Eye disorders	**1**	**0**	**1**	2.0	4.6
Unlikely	1	0	1		
Gastrointestinal disorders	**0**	**7**	**7**	13.7	31.9
Not Related		7	7		
General disorders and administration site conditions	**2**	**7**	**9**	17.6	41
Not Related	0	2	2		
Unlikely	0	3	3		
Probably	1	1	2		
Possibly	1	0	1		
Related	0	1	1		
Infections and infestations	**2**	**2**	**4**	7.8	18.2
Not Related	2	2	4		
Metabolism and nutrition disorders	**0**	**1**	**1**	2.0	4.6
Unlikely	0	1	1		
Nervous system disorders	**3**	**3**	**6**	11.8	27.4
Not Related	3	1	4		
Unlikely	0	2	2		
Psychiatric disorders	**1**	**0**	**1**	2.0	4.6
Not related	1	0	1		
Reproductive system and breast disorders	**1**	**0**	**1**	2.0	4.6
Not Related	1	0	1		
Respiratory, thoracic and mediastinal disorders	**0**	**1**	**1**	2.0	4.6
Not Related	0	1	1		
Skin and subcutaneous tissue disorders	**7**	**11**	**18**	35.3	82.1
Not Related	5	6	11		
Unlikely	2	3	5		
Possibly	0	2	2		
Total	**19**	**32**	**51**	**100.0**	**232.6**

Administration site conditions per treatment regimen and relationship

		ATx201	Vehicle	Total
Application site erythema		**1**	**0**	**1**
	Probably	1	0	1
Application site pruritus		**0**	**1**	**1**
	Possibly	0	1	1
Burning sensations		**2**	**2**	**4**
	Unlikely	0	1	1
	Probably	1	0	1
	Related	1	1	2
Total		**3**	**3**	**6**

Note. AE: Adverse event; qd: once-daily; SAF: Safety analysable population; SOC: System organ class; bid: twice-daily.

Table S4. Background information on *S. aureus* and MRSA clinical isolates included in this study. n.a.: information was not available. CF: cystic fibrosis. PVL: Panton-Valentine leukocidin.

***S. aureus* Isolates ID**	**MLST-SCC*mec***	**Spa type**	**Collection site**	**Other information**
S. aureus ATCC 29213	-	-	-	CLSI reference strain
S. aureus Newman	ST8 - IV	t008		n.a.
MRSA 01	ST30-IV	t019	Skin abscess	USA300
MRSA 02	ST1-IV	t127	n.a	South East Pacific clone
MRSA 03	ST772-V	t657	n.a	USA400. PVL positive.
MRSA 04	ST130-XI	t843	n.a	Bengal clone
MRSA 05	CC97 – 5C2 (V)	n.a.	n.a	n.a.
MRSA 06	ST398	n.a.	n.a	n.a.
MRSA 07	ST8	n.a.	n.a	n.a.
MRSA 08	ST45	n.a.	n.a	USA300
MRSA 09	ST22 - IV	n.a.	n.a	USA600
MRSA 10	ST36 - II	n.a.	n.a	Epidemic MRSA
MRSA 11	ST8 - IV	t008	n.a	n.a.
EEFIC 01	CC123	t171	n.a	n.a.
EEFIC 02	CC123	t171	n.a	n.a.
MRSA 12	CC80	t044	n.a	n.a.
MRSA 13	CC80	t044	n.a	n.a.
MSSA 01	CC1	t127	n.a	n.a.
MSSA 02	CC1	t127	n.a	n.a.
MRSA 14	CC22	t2006	n.a	n.a.
MRSA 15	CC30	t166	n.a	n.a.
MRSA 16	CC30	t019	n.a	n.a.
MRSA 17	CC509	t375	n.a	n.a.
MRSA 18	CC22	t1328	n.a	Mutation in urease gene
MRSA 19	CC8	n.a.	n.a	MRSA10 with mutation in urease gene
MRSA 20	ST239	n.a.	n.a	CA-MRSA
MRSA 21	CC398	t899	n.a	Hospital clone from Turkey and Vietnam
MRSA 22	ST5	t002	n.a	LA-MRSA
MRSA 23	ST5	t002	n.a	Diverse clone
MRSA 24	ST30	n.a.	n.a	South West Pacific clone, CA-MRSA
MRSA 25	ST1	n.a.	n.a	Diverse clone
MRSA 26	ST59	n.a.	n.a	Far East clone
MRSA 27	ST80	n.a.	n.a	Mediterranean clone, CA-MRSA
MRSA28	ST45	n.a.	n.a	Diverse clone
MRSA 29	n.a.	t024	n.a	Danish MRSA isolate related with USA300
MRSA 30	CC22	n.a.	n.a	PVL positive
MRSA 31	CC398	t011	n.a	n.a.
MRSA 32	CC30	t005	n.a	PVL negative
MRSA 33	n.a.	t2872	n.a	mecC variant
MRSA 34	ST1327	t021	n.a	n.a.
MRSA 35	ST36	t688	n.a	n.a.
MRSA 36	ST30	t002	n.a	n.a.
MRSA 37	ST5	t304	n.a	n.a.
MRSA 38	ST149-SLV	t579	n.a	n.a.

MRSA 39	ST8	t024	n.a	n.a.
MRSA 40	ST2626	t034	n.a	n.a.
MRSA 41	ST8	t005	n.a	n.a.
MRSA 42	ST398	t2872	n.a	n.a.
MRSA 43	ST80	t044	n.a	n.a.
MRSA 44	ST80	t1028	n.a	n.a.
MRSA 45	ST46	t2988	n.a	n.a.
MRSA 46	ST88	t1855	n.a	n.a.
MRSA 47	ST225	t003	n.a	n.a.
MRSA 48	ST6	t304	n.a	n.a.
MRSA 49	ST125	t067	n.a	n.a.
MRSA 50	ST88	t690	n.a	n.a.
MRSA 51	ST1327	t005	n.a	n.a.
MRSA 52	ST8	t008	n.a	n.a.
MRSA 53	ST22	t032	n.a	n.a
MRSA 54	ST45	t050	n.a	n.a
MRSA 55	ST8	t3980	n.a	n.a
MRSA 56	ST45	t1510	n.a	n.a
MRSA 57	ST859	t3310	n.a	n.a
MRSA 58	ST630	t4549	n.a	n.a
MRSA 59	ST1	t127	n.a	n.a
MRSA 60	ST8	t008	n.a	n.a
MRSA 61	ST80	t044	n.a	n.a
MRSA 62	ST30	t019	n.a	n.a
MRSA 63	ST30	t019	n.a	n.a
MRSA 64	ST6	t304	n.a	n.a
MRSA 65	ST1	t127	n.a	n.a
MRSA 66	ST72	t148	n.a	n.a
MRSA 67	ST149	t002	n.a	n.a
MRSA 68	ST1633	t355	n.a	n.a
MRSA 69	ST8	t024	n.a	n.a
MRSA 70	ST72	t3169	n.a	n.a
MRSA 71	ST88	t1814	n.a	n.a
MRSA 72	ST1	t127	n.a	n.a
MRSA 73	ST5-SLV	t002	n.a	n.a
MRSA 74	ST97	t267	n.a	n.a
MRSA 75	ST8	t008	n.a	n.a
MRSA 76	ST5	t088	n.a	n.a
MRSA 77	ST772	t1839	n.a	n.a
MRSA 78	ST6	t304	n.a	n.a
MRSA 79	ST22	t025	n.a	n.a
MRSA 80	ST22-SLV	t852	n.a	n.a
MRSA 81	ST6	t304	n.a	n.a
MRSA 82	ST1	t127	n.a	n.a
MRSA 83	ST88	t1814	n.a	n.a
MRSA 27b	ST80	t044	n.a	n.a
MRSA 84	ST6	t304	n.a	n.a
MRSA 85	ST8	t024	n.a	n.a
MRSA 86	ST5	t002	n.a	n.a
MRSA 87	ST45	t116	n.a	n.a
MRSA 88	ST30	t019	n.a	n.a
MRSA 89	ST59	t437	n.a	n.a
MRSA 90	ST149	t002	n.a	n.a

MRSA 91	ST737	t005	n.a	n.a
MRSA 92	ST1	t127	n.a	n.a
MRSA 93	ST1	t1853	n.a	n.a
MRSA 94	ST1456	t019	n.a	n.a
MRSA 95	ST15-SLV	t084	n.a	n.a
MRSA 96	ST2626	t579	n.a	n.a
MRSA 97	ST5	t548	n.a	n.a
MRSA 98	ST188	t189	n.a	n.a
MRSA 99	ST45	t015	n.a	n.a
MRSA 100	ST8	t008	n.a	n.a
MRSA 101	ST1	t127	n.a	n.a
MRSA 102	ST1472	t665	n.a	n.a
MRSA 103	ST149	t002	n.a	n.a
MRSA 104	ST5	t002	n.a	n.a
MRSA 105	ST508	t3219	n.a	n.a
MRSA 106	ST22	t309	n.a	n.a
MRSA 107	ST772	t5414	n.a	n.a
MRSA 108	ST8	t024	n.a	n.a
MRSA 109	ST121	t4214	n.a	n.a
MRSA 110	ST130	t5090	n.a	n.a
MRSA 111	ST5	t688	n.a	n.a
MRSA 112	ST8	t008	n.a	n.a
MRSA 113	ST8	t008	n.a	n.a
K000796	n.a	n.a	Blood culture	n.a
K115688	n.a	n.a	Peritoneal fluid	n.a
K000866	n.a	n.a	Blood culture	n.a
K000864	n.a	n.a	Blood culture	n.a
K000863	n.a	n.a	Blood culture	n.a
K115689	n.a	n.a	Peritoneal fluid	n.a
K000772	n.a	n.a	Blood culture	n.a
K115498	n.a	n.a	Blood culture	n.a
R000024	n.a	n.a	Mammae abscess	n.a
R000020	n.a	n.a	Skin swab	n.a
R000019	n.a	n.a	Skin swab	n.a
U115579	n.a	n.a	Urine from catheter	n.a
U115370	n.a	n.a	Urine	n.a
U114660	n.a	n.a	Urine	n.a
D115584	n.a	n.a	Fluid from scrotum	n.a
E115740	n.a	n.a	Sputum sample	n.a
E116152	n.a	n.a	Sputum sample	n.a
E115810	n.a	n.a	Sputum sample	n.a
T115628	n.a	n.a	Skin swab	n.a
T000274	n.a	n.a	Skin swab	n.a
T115691	n.a	n.a	Skin swab	n.a
T115903	n.a	n.a	Skin swab	n.a
T116122	n.a	n.a	Wound swab	n.a
T115015	n.a	n.a	Skin swab	n.a
C115273	n.a	n.a	CF Sputum sample	n.a
C000040	n.a	n.a	CF Sputum sample	n.a
C115690	n.a	n.a	CF Sputum sample	n.a
C115561	n.a	n.a	CF Sputum sample	n.a
C 115445	n.a	n.a	CF Sputum sample	n.a
C115263	n.a	n.a	CF Sputum sample	n.a

C115303	n.a	n.a	CF Sputum sample	n.a
C115268	n.a	n.a	CF Sputum sample	n.a
C115295	n.a	n.a	CF Sputum sample	n.a
C115242	n.a	n.a	CF Sputum sample	n.a
C115427	n.a	n.a	CF Sputum sample	n.a
C000041	n.a	n.a	CF Sputum sample	n.a
E5-1048654	n.a	n.a	Wound	n.a
9-2955245	n.a	n.a	Wound	n.a
E5-1046019	n.a	n.a	Wound	n.a
E5-1046020	n.a	n.a	Wound	n.a
E5-1047585	n.a	n.a	Wound	n.a
E5-1038294	n.a	n.a	Wound	n.a
E5-1035779	n.a	n.a	Wound	n.a
9-1862936	n.a	n.a	Wound	n.a
E5-1033091	n.a	n.a	Wound	n.a
9-2642166	n.a	n.a	Wound	n.a
9-2642158	n.a	n.a	Wound	n.a
E5-1035775	n.a	n.a	Wound	n.a
E5-1029558	n.a	n.a	Skin	n.a
E5-1038279	n.a	n.a	Skin	n.a
E5-1039697	n.a	n.a	Skin	n.a
E5-1041979	n.a	n.a	Skin	n.a
E5-1035284	n.a	n.a	Skin	n.a
E5-1030469	n.a	n.a	Skin	n.a
E5-1030472	n.a	n.a	Skin	n.a
E5-1041977	n.a	n.a	Wound	n.a
E5-1041987	n.a	n.a	Wound	n.a
E5-1039684	n.a	n.a	Wound	n.a
E5-1041980	n.a	n.a	Wound	n.a
E5-1033088	n.a	n.a	Wound	n.a
E5-1035277	n.a	n.a	Wound	n.a
E5-1046096	n.a	n.a	Podning	n.a
E5-1046085	n.a	n.a	Wound	n.a
9-2625962	n.a	n.a	Wound	n.a
E5-1043668	n.a	n.a	Skin	n.a
E5-1048428	n.a	n.a	Abcess	n.a
E5-1047924	n.a	n.a	Wound	n.a
E5-1047606	n.a	n.a	Wound	n.a
E5-1046070	n.a	n.a	Wound	n.a
E5-1046298	n.a	n.a	Wound	n.a
E5-1046296	n.a	n.a	Wound	n.a
E5-1046297	n.a	n.a	Wound	n.a
E5-1043184	n.a	n.a	Wound	n.a
E5-1038286	n.a	n.a	Wound	n.a
E5-1037958	n.a	n.a	Wound	n.a
E5-1037971	n.a	n.a	Wound	n.a
E5-1033076	n.a	n.a	Wound	n.a
E5-1029252	n.a	n.a	Wound	n.a
E5-1030440	n.a	n.a	Wound	n.a
E5-1030482	n.a	n.a	Wound	n.a
E5-1046074	n.a	n.a	Wound	n.a
E5-1048204	n.a	n.a	Lung	n.a
E5-1048670	n.a	n.a	Abcess	n.a

E5-1046039	n.a	n.a	Wound	n.a
E5-1045179	n.a	n.a	Wound	n.a
E5-1046723	n.a	n.a	Vagina	n.a

Table S5. Full list of inclusion and exclusion criteria.

Inclusion criteria
1. Signed and dated informed consent has been obtained 2. Age 18-70 years 3. Male or female 4. Female subjects of childbearing potential had to be confirmed not pregnant by a negative urine pregnancy test prior trial treatment 5. Female subjects of childbearing potential had to be willing to use effective contraceptive at trial entry until completion 6. Male subjects had to agree to use adequate contraception for the duration of the trial 7. Localized disease where two individual lesions each covering an area between 10-200 cm^2 and where each individual lesion has an IGA score between 1-4 and is colonized by *S. aureus* with at least 1,000 CFU/ cm^2. 8. Additional localized lesion of area between 10-200 cm^2 and where the individual lesion has an IGA score between 1 and 4.
Exclusion criteria
1. Clinically relevant abnormalities in the laboratory testing, vital signs, ECG (Part 1 only) or physical examination unless considered clinically irrelevant for the scope of the trial by the investigator. 2. Presence of any skin condition (scars, tattoos, etc.) that would interfere with the placement of study medication. 3. History of irritation to topical products. 4. Current acute or chronic disease unless considered clinically irrelevant for the scope of the trial by the investigator. 5. Relevant history of malignancy, of renal, hepatic, cardiovascular, respiratory, gastrointestinal, musculoskeletal, skin (particularly at the site of drug application), hematological, endocrine or neurological diseases that may interfere with the aim of the study. 6. Positive HIV serology or evidence of active hepatitis. 7. Ascertained or presumptive hypersensitivity to the active principle and/or formulations ingredients of the study drugs (test, reference). 8. History of drug or alcohol abuse (>2 drinks/day, defined according to USDA Dietary Guidelines 2005). 9. Blood donations during 6 weeks prior to this study or planned within 6 weeks after the last blood withdrawal. 10. Subject considered unable or unlikely (per investigator judgement) to comply with safety and PK profiling requirements (follow-up visits). 11. Subjects who are pregnant (as determined by a positive pregnancy test at the screening Visit) or lactating. 12. Participation in another clinical trial with an investigational drug within 4 weeks before screening. 13.Treatment with antibiotics (systemic or topical) within the past 2 months taken for systemic application, and 4 weeks use of topical antibiotics prior entering the study and use of those during the study.

14. Treatment with cyclosporins within the last four weeks, use of methotrexate or mycophenolate mofertil within the last eight weeks, and biologicals within 5 times the biological half-life prior to entering the study.
15. Treatment with topical (dermatological) steroids and calcineurin inhibitors 1 week prior to start of treatment and during the study.
16. Treatment with systemic steroids within the past month and during the study.
17. Use of disinfectant soaps within 1 week before screening and during the study treatment period.

CLINICAL TRIAL ETHICAL APPROVAL

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Sitzung der Ethik-Kommission am 04.10.2016, TOP 13:

EK Nr: 1947/2016
EudraCT Nr: 2016-003501-33
Projekttitel: Eine randomisierte, doppelblinde, Placebo kontrollierte Studie in 2 Phasen um die Sicherheit und die Wirksamkeit von ATx201 als topisches Antibiotikum in einem Prüfzentrum zu überprüfen (DECOLAD).
Antragsteller/in: Herr Dr. rer. nat. Benjamin Reutterer
Institution: KKS
Sponsor: AntibioTx ApS

Teilnehmende Prüfzentren:

Ethik-Kommission	Prüfzentrum	Prüfärztin/arzt
Ethikkommission der Medizinischen Universität Wien	Universitätsklinik für Klinische Pharmakologie	Herr Assoc. Prof. Priv.-Doz. Dr. Markus Zeitlinger

Die Stellungnahme der Ethik-Kommission erfolgt aufgrund folgender eingereichter Unterlagen:

Dokument	Name	Version	Datum
Fallbericht-Formular (CRF)	DECOLAD - eCRF - V0.1 - 25.08.2016	0.1	25.08.2016
Conflict of Interest	DECOLAD - Conflict of Interest Markus Zeitlinger - 23.08.2016	1.0	23.08.2016
Covering Letter	DECOLAD - cover letter EK - 07.09.2016	1.0	07.09.2016
Lebenslauf (CV)	DECOLAD - CV Markus Zeitlinger - 30.06.2016	1.0	30.06.2016
	DECOLAD - CV Martin Bauer - 30.06.2016	1.0	30.06.2016
EudraCT-Formular	DECOLAD - EudraCT Form signed - 07.09.2016	1.0	07.09.2016
Versicherungsbestätigung	DECOLAD - Insurance Zürich 28092016	1.0	28.09.2016
	DECOLAD - Insurance Lloyds - Wording (CTIS2015A)	1.0	07.09.2016
	DECOLAD - Insurance Lloyds - Wording (CTIS2015B)	1.0	07.09.2016
	DECOLAD - Insurance Lloyds - Wording (CTIS2015C)	1.0	07.09.2016
	DECOLAD - Insurance Certificate Lloyds-30.08.2016	1.0	30.08.2016
	DECOLAD - Insurance policy Lloyds-30.08.2016	1.0	30.08.2016

Prüferinformation (Investigator's Brochure)	DECOLAD - Investigators Brochure ATx201 - V.1 signed - 06.09.2016	1.0	06.09.2016
Sonstige	Schreiben EK 20.09.2016	1.0	20.09.2016
	Interne Leistungsverrechnung 1947_2016	1.0	19.09.2016
	DECOLAD - Patient diary - IMP once daily v.1.0 - 05.09.2016	1.0	05.09.2016
	DECOLAD - Patient diary - IMP twice daily - V1.0 - 05.09.2016	1.0	05.09.2016
	DECOLAD - Letter of Authorization signed - 29.08.2016	1.0	29.08.2016
Patienteninformation	DECOLAD - Informed Consent - Patients - V.1.0 - 05.09.2016	1.0	05.09.2016
	DECOLAD - Informed Consent - Volunteers Validation - V.1.0 - 05.09.2016	1.0	05.09.2016
	DECOLAD - Informed Consent - Volunteers Treatment - V1.0. - 05.09.2016	1.0	05.09.2016
Studienprotokoll (Prüfplan)	DECOLAD - Signature page KKS - Clinical Study Protocol - 06.09.2016	1.1	06.09.2016
	DECOLAD - Signature page PI - Clinical Study Protocol - 06.09.2016	1.1	06.09.2016
	DECOLAD - Signature page Sponsor - Clinical Study Protocol - 06.09.2016	1.1	06.09.2016
	DECOLAD - Signature page Statistician - Clinical Study Protocol - 06.09.2016	1.1	06.09.2016
	DECOLAD - Clinical Study Protocol - Version 1.1 - 05.09.2016	1.1	05.09.2016

Die Kommission fasst folgenden Beschluss (mit X markiert):

☒	Die unten bezeichneten Punkte des Antrages sind entweder noch unerledigt bzw sollten von den Antragstellern geändert/ nachgereicht werden. Nach entsprechender Vorlage/Erledigung kann auch vor der nächsten Ethik-Kommissions Sitzung ein endgültig positiver Beschluss ausgefertigt werden. Der Antrag wird in der nächsten Sitzung der Kommission nicht mehr behandelt. Achtung: Werden die geforderten Unterlagen von den Antragstellern nicht innerhalb von 3 Sitzungsperioden (ab Datum dieser Sitzung) nachgereicht, gilt der Antrag ohne weitere Benachrichtigung als zurückgezogen und muss gegebenenfalls als Neuantrag eingereicht werden.

Ergänzende Kommentare der Sitzung am 04.10.2016:

Zum Prüfplan:
Das externe Gutachten ist zum Sitzungszeitpunkt noch ausständig.
Die Ethik-Kommission ersucht um eine Begründung im Protokoll, warum als Lokalisation für die Behandlung und damit für die Biopsien die Unterarme gewählt wurden. Wären weniger sichtbare Körperstellen für die Biopsien auch möglich?

Punkt 9.5.10 "skin sampling": Die Technik des Abstrichs sollte genauer definiert werden. (Wie wird garantiert, dass die Quantifizierung der Keimzahl der Kulturen vergleichbar sind?)

Zum Antrag:
Die Kurzfassung unter Punkt 7.2 ist ausführlicher zu gestalten und sollte folgendes beinhalten:

Rechtfertigung, Relevanz, Design, Maßnahmen und Vorgehensweise.
Die Angaben zur Biometrie sind zu ergänzen.

Zur Statistik:
Die Methode der Randomisierung ist im Protokoll zu beschreiben.
Laut Tabelle 5 im Protokoll ist für Phase II der Studie ein Cross-over Design geplant, dies sollte im Protokoll (und evtl. Titel) explizit erwähnt werden. Zudem ist die geplante Analyse (Abschnitt 12.6.2) mit dem Fisher exact test für einen Cross-over Versuch nicht geeignet, da dieser die Abhängigkeit der Messungen am gleichen Patienten nicht berücksichtigt. Aus diesem Grund ist auch die Fallzahlbegründung, die auf einem Chi-Quadrat Test beruht, nicht zutreffend.

Zur Patienteninformation:
Probanden/Validation:
Punkt 2: Bei der Erwähnung der Validierungsgruppe sollte hinzugefügt werden, dass dies die Gruppe ist, an der die Probanden teilnehmen. Es sollte auch erklärt werden, was der Zweck dieser Phase ist und wofür die abgenommenen Proben verwendet werden.
Punkt 12: Es ist zu ergänzen, dass bei vorzeitigem Rücktritt von der Studie ein anteiliger Betrag erstattet wird.
Punkt 13: Es fehlen die Kontaktdaten der Studienärzte.
Punkt 16: Die Information hat insgesamt 6 Seiten, nicht 7 - die Angabe der Seitenzahl im Text ist zu korrigieren. Die Formulierung "Ich bin ...über ATx201...aufgeklärt worden" trifft nicht zu, in diesem Studienteil wird die Substanz überhaupt nicht verabreicht.

Probanden/Treatment:
Punkt 2: Der Stufenablauf ist nicht sehr klar beschrieben. Es ist unklar, ob in den beiden Absätzen beginnend mit "Danach wird Ihre allgemeine Krankengeschichte erhoben..." und "Während des ersten Besuchs nach Einschluss..." vom gleichen Besuch die Rede ist oder von zwei unterschiedlichen Besuchen. Dies muss klar hervorgehen und falls es sich um zwei Besuche handelt, muss angegeben werden, in welchem Abstand diese stattfinden. Was ist mit den "bereits oben beschriebenen Zeitpunkten" gemeint? Die Zeitpunkte der Anwendung der Prüfsubstanz? Dies sollte explizit angegeben werden. Anstelle von "Am darauffolgenden Tag" sollte es besser heißen "an Tag 8". Der Begriff "lineare Sonden" sollte erklärt (oder gestrichen) werden. Es sollte angegeben werden, wie lange die Mikrodialyse dauert. Es ist anzuführen, wie viel Blut bei den Besuchen jeweils abgenommen wird.
Punkt 13: Es ist zu ergänzen, dass bei vorzeitigem Rücktritt von der Studie ein anteiliger Betrag erstattet wird.
Punkt 14: Es fehlen die Kontaktdaten der Studienärzte.

Patienten:
Punkt 3: Es ist anzuführen, wie viel Blut jeweils bei den Besuchen abgenommen wird.
Punkt 5: Da die Patienten das Medikament ja nur an einem eingeschränkten Areal verwenden, ist die Angabe zum Nutzen stark zu relativieren.
Punkt 14: Es ist zu ergänzen, dass bei vorzeitigem Rücktritt von der Studie ein anteiliger Betrag erstattet wird.
Punkt 15: Es fehlen die Kontaktdaten der Studienärzte.

Zur Versicherung:
Es wurde die Versicherungsbestätigung der Lloyds/QBE Syndicate (zu Pol. CTISQBE16175), datiert mit 30.8.2016, beurteilt. Diese ist akzeptabel für insgesamt 81 Studienteilnehmer. Zusätzlich wurde auch die Versicherungsbestätigung der Zürich Versicherung (Rahmenversicherung der MedUni Wien) vorgelegt, diese wurde noch nicht beurteilt. Die Ethik-Kommission ersucht um Stellungnahme, warum zwei verschiedene Versicherungsdokumente eingereicht wurden, in den Teilnehmerinformationen ist nur die Zürich Versicherung angeführt.

Die Ethik-Kommission ersucht die Antragsteller, bei der Wiedervorlage von geänderten Unterlagen ein Exemplar mit hervorgehobenen Änderungen beizulegen.

"CLOCKSTOP": Die in §41a AMG genannte Frist wird ab dem Sitzungstag bis zum Einlangen der in diesem Votum geforderten Informationen gehemmt.

Die Ethik-Kommision geht - rechtlich unverbindlich - davon aus, daß es sich um eine klinische Prüfung gemäß AMG handelt.

Die aktuelle Mitgliederliste der Ethik-Kommission ist unter der Adresse http://ethikkommission.meduniwien.ac.at/ethik-kommission/mitglieder/ abrufbar. Mitglieder der Ethik-Kommission, die für diesen Tagesordnungspunkt als befangen anzusehen waren und daher laut Geschäftsordnung an der Entscheidungsfindung/Abstimmung nicht teilgenommen haben: Frau Dr. Christa Firbas, Herr Martin Brunner, Frau Dr. Brigitte Blöchl-Daum, Herr Martin Bauer, Frau Brigitte Litschauer

ACHTUNG: Unter Berücksichtigung der "ICH-Guideline for Good Clinical Practice" gilt dieser Beschluss **ein Jahr ab Datum der Ausstellung**. Gegebenenfalls hat der Antragsteller eine Verlängerung der Gültigkeit rechtzeitig zu beantragen.

Dieses Votum ist für berechtigte Benutzer/innen in digitaler Form unter der Adresse https://ekmeduniwien.at/vote/10232/download/ abrufbar.

SIGNATURINFORMATIONEN	**Unterzeichner**	Dr. Jürgen Zezula
	Datum/Zeit-UTC	2016-10-10T11:53:45Z
	Prüfinformation	Informationen zur Prüfung der elektronischen Signatur finden Sie unter: https://www.signaturpruefung.gv.at

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ethikkommission.meduniwien.ac.at

Votum:

EK Nr: 1947/2016
EudraCT Nr: 2016-003501-33
Projekttitel: Eine randomisierte, doppelblinde, Placebo kontrollierte Studie in 2 Phasen um die Sicherheit und die Wirksamkeit von ATx201 als topisches Antibiotikum in einem Prüfzentrum zu überprüfen (DECOLAD).
Antragsteller/in: Herr Dr. rer. nat. Benjamin Reutterer
Institution: KKS
Sponsor: AntibioTx ApS

Teilnehmende Prüfzentren:

Ethik-Kommission	Prüfzentrum	Prüfärztin/arzt
Ethikkommission der Medizinischen Universität Wien	Universitätsklinik für Klinische Pharmakologie	Herr Assoc. Prof. Priv.-Doz. Dr. Markus Zeitlinger

Die Stellungnahme der Ethik-Kommission erfolgt aufgrund folgender eingereichter Unterlagen:

Dokument	Name	Version	Datum
Fallbericht-Formular (CRF)	DECOLAD - eCRF - V0.1 - 25.08.2016	0.1	25.08.2016
Conflict of Interest	DECOLAD - Conflict of Interest Markus Zeitlinger - 23.08.2016	1.0	23.08.2016
Covering Letter	DECOLAD - covering letter 1.0 30112016	1.0	30.11.2016
	DECOLAD - cover letter EK - 27.10.2016	1.0	27.10.2016
	DECOLAD - cover letter EK - 07.09.2016	1.0	07.09.2016
Lebenslauf (CV)	DECOLAD - CV Markus Zeitlinger - 30.06.2016	1.0	30.06.2016
	DECOLAD - CV Martin Bauer - 30.06.2016	1.0	30.06.2016
EudraCT-Formular	DECOLAD - EudraCT Form signed - 07.09.2016	1.0	07.09.2016
Versicherungsbestätigung	DECOLAD - Insurance Zürich 28092016	1.0	28.09.2016
	DECOLAD - Insurance Lloyds - Wording (CTIS2015A)	1.0	07.09.2016
	DECOLAD - Insurance Lloyds - Wording (CTIS2015B)	1.0	07.09.2016
	DECOLAD - Insurance Lloyds - Wording (CTIS2015C)	1.0	07.09.2016
	DECOLAD - Insurance Certificate Lloyds-30.08.2016	1.0	30.08.2016

	DECOLAD - Insurance policy Lloyds-30.08.2016	1.0	30.08.2016
Prüferinformation (Investigator's Brochure)	DECOLAD - Investigators Brochure ATx201 - V.1 signed - 06.09.2016	1.0	06.09.2016
Sonstige	Schreiben EK 20.09.2016	1.0	20.09.2016
	Interne Leistungsverrechnung 1947_2016	1.0	19.09.2016
	DECOLAD - Patient diary - IMP once daily v.1.0 - 05.09.2016	1.0	05.09.2016
	DECOLAD - Patient diary - IMP twice daily - V1.0 - 05.09.2016	1.0	05.09.2016
	DECOLAD - Letter of Authorization signed - 29.08.2016	1.0	29.08.2016
Patienteninformation	DECOLAD - Informed Consent - Patients - V.2.0-19.10.2016 trackchange	2.0	19.10.2016
	DECOLAD - Informed Consent - Patients - V.2.0-19.10.2016	2.0	19.10.2016
	DECOLAD - Informed Consent - Volunteers Treatment - V2.0. - 19.10.2016	2.0	19.10.2016
	DECOLAD - Informed Consent - Volunteers Treatment - V2.0. - 19.10.2016_trackchange	2.0	19.10.2016
	DECOLAD - Informed Consent - Volunteers Validation - V.2.0 - 19.10.2016 trackchange	2.0	19.10.2016
	DECOLAD - Informed Consent - Volunteers Validation - V.2.0 - 19.10.2016	2.0	19.10.2016
	DECOLAD - Informed Consent - Patients - V.1.0 - 05.09.2016	1.0	05.09.2016
	DECOLAD - Informed Consent - Volunteers Validation - V.1.0 - 05.09.2016	1.0	05.09.2016
Studienprotokoll (Prüfplan)	DECOLAD - Clinical Study Protocol - Version 3.0 17.11.2016_track change	3.0	17.11.2016
	DECOLAD - Clinical Study Protocol - Version 3.0 17.11.2016	3.0	17.11.2016
	DECOLAD - Clinical Study Protocol - Version 2.0 - 24.10.2016 TC	2.0	24.10.2016
	DECOLAD - Clinical Study Protocol - Version 2.0 - 24.10.2016	2.0	24.10.2016
	DECOLAD - Signature page KKS - Clinical Study Protocol - 06.09.2016	1.1	06.09.2016
	DECOLAD - Signature page PI - Clinical Study Protocol - 06.09.2016	1.1	06.09.2016
	DECOLAD - Signature page Sponsor - Clinical Study Protocol - 06.09.2016	1.1	06.09.2016
	DECOLAD - Signature page Statistician - Clinical Study Protocol - 06.09.2016	1.1	06.09.2016
	DECOLAD - Clinical Study Protocol - Version 1.1 - 05.09.2016	1.1	05.09.2016

Die Kommission fasst folgenden Beschluss (mit X markiert):

☒	Es besteht kein Einwand gegen die Durchführung der Studie.

Ergänzende Kommentare der Sitzung am 04.10.2016:

Zum Prüfplan:
Das externe Gutachten ist zum Sitzungszeitpunkt noch ausständig.
Die Ethik-Kommission ersucht um eine Begründung im Protokoll, warum als Lokalisation für die Behandlung und damit für die Biopsien die Unterarme gewählt wurden. Wären weniger sichtbare Körperstellen für die Biopsien auch möglich?
Punkt 9.5.10 "skin sampling": Die Technik des Abstrichs sollte genauer definiert werden. (Wie wird garantiert, dass die Quantifizierung der Keimzahl der Kulturen vergleichbar sind?)

Zum Antrag:
Die Kurzfassung unter Punkt 7.2 ist ausführlicher zu gestalten und sollte folgendes beinhalten: Rechtfertigung, Relevanz, Design, Maßnahmen und Vorgehensweise.
Die Angaben zur Biometrie sind zu ergänzen.

Zur Statistik:
Die Methode der Randomisierung ist im Protokoll zu beschreiben.
Laut Tabelle 5 im Protokoll ist für Phase II der Studie ein Cross-over Design geplant, dies sollte im Protokoll (und evtl. Titel) explizit erwähnt werden. Zudem ist die geplante Analyse (Abschnitt 12.6.2) mit dem Fisher exact test für einen Cross-over Versuch nicht geeignet, da dieser die Abhängigkeit der Messungen am gleichen Patienten nicht berücksichtigt. Aus diesem Grund ist auch die Fallzahlbegründung, die auf einem Chi-Quadrat Test beruht, nicht zutreffend.

Zur Patienteninformation:
Probanden/Validation:
Punkt 2: Bei der Erwähnung der Validierungsgruppe sollte hinzugefügt werden, dass dies die Gruppe ist, an der die Probanden teilnehmen. Es sollte auch erklärt werden, was der Zweck dieser Phase ist und wofür die abgenommenen Proben verwendet werden.
Punkt 12: Es ist zu ergänzen, dass bei vorzeitigem Rücktritt von der Studie ein anteiliger Betrag erstattet wird.
Punkt 13: Es fehlen die Kontaktdaten der Studienärzte.
Punkt 16: Die Information hat insgesamt 6 Seiten, nicht 7 - die Angabe der Seitenzahl im Text ist zu korrigieren. Die Formulierung "Ich bin ...über ATx201...aufgeklärt worden" trifft nicht zu, in diesem Studienteil wird die Substanz überhaupt nicht verabreicht.

Probanden/Treatment:
Punkt 2: Der Stufenablauf ist nicht sehr klar beschrieben. Es ist unklar, ob in den beiden Absätzen beginnend mit "Danach wird Ihre allgemeine Krankengeschichte erhoben..." und "Während des ersten Besuchs nach Einschluss..." vom gleichen Besuch die Rede ist oder von zwei unterschiedlichen Besuchen. Dies muss klar hervorgehen und falls es sich um zwei Besuche handelt, muss angegeben werden, in welchem Abstand diese stattfinden. Was ist mit den "bereits oben beschriebenen Zeitpunkten" gemeint? Die Zeitpunkte der Anwendung der Prüfsubstanz? Dies sollte explizit angegeben werden. Anstelle von "Am darauffolgenden Tag" sollte es besser heißen "an Tag 8". Der Begriff "lineare Sonden" sollte erklärt (oder gestrichen) werden. Es sollte angegeben werden, wie lange die Mikrodialyse dauert. Es ist anzuführen, wie viel Blut bei den Besuchen jeweils abgenommen wird.
Punkt 13: Es ist zu ergänzen, dass bei vorzeitigem Rücktritt von der Studie ein anteiliger Betrag erstattet wird.
Punkt 14: Es fehlen die Kontaktdaten der Studienärzte.

Patienten:
Punkt 3: Es ist anzuführen, wie viel Blut jeweils bei den Besuchen abgenommen wird.
Punkt 5: Da die Patienten das Medikament ja nur an einem eingeschränkten Areal verwenden, ist die Angabe zum Nutzen stark zu relativieren.
Punkt 14: Es ist zu ergänzen, dass bei vorzeitigem Rücktritt von der Studie ein anteiliger Betrag erstattet wird.
Punkt 15: Es fehlen die Kontaktdaten der Studienärzte.

Zur Versicherung:
Es wurde die Versicherungsbestätigung der Lloyds/QBE Syndicate (zu Pol. CTISQBE16175), datiert mit 30.8.2016, beurteilt. Diese ist akzeptabel für insgesamt 81 Studienteilnehmer. Zusätzlich wurde auch die Versicherungsbestätigung der Zürich Versicherung (Rahmenversicherung der MedUni Wien) vorgelegt, diese wurde noch nicht beurteilt. Die Ethik-Kommission ersucht um Stellungnahme, warum zwei verschiedene Versicherungsdokumente eingereicht wurden, in den Teilnehmerinformationen ist nur die Zürich Versicherung angeführt.

Die Ethik-Kommission ersucht die Antragsteller, bei der Wiedervorlage von geänderten Unterlagen ein Exemplar mit hervorgehobenen Änderungen beizulegen.

Ergänzende Kommentare:
Nachtrag vom 1. Dezember 2016:
Die Antragsteller legen am 30.11.2016 überarbeitete Unterlagen vor, die von der Ethik-Kommission akzeptiert werden. Es liegen gültige Versicherungsunterlagen vor.
Die Antragsteller werden darauf hingewiesen, dass im Falle von Protokolländerungen die Versicherung zu informieren ist. Bei Fehlen einer automatischen Verlängerung des Versicherungsvertrages ist rechtzeitig eine Versicherungsbestätigung über die Verlängerung vorzulegen.
Begründung der Befürwortung des Antrages durch die Ethik-Kommission: Es handelt sich bei dem vorliegenden Projekt um eine relevante Fragestellung, die mit geeigneter Methodik beantwortet werden soll. Die vorgenommene Bewertung des Nutzen/Risikoverhältnisses wird von der Ethik-Kommission anerkannt.

Die Ethik-Kommision geht - rechtlich unverbindlich - davon aus, daß es sich um eine klinische Prüfung gemäß AMG handelt.

Die aktuelle Mitgliederliste der Ethik-Kommission ist unter der Adresse http://ethikkommission.meduniwien.ac.at/ethik-kommission/mitglieder/ abrufbar. Mitglieder der Ethik-Kommission, die für diesen Tagesordnungspunkt als befangen anzusehen waren und daher laut Geschäftsordnung an der Entscheidungsfindung/Abstimmung nicht teilgenommen haben: Frau Dr. Christa Firbas, Herr Martin Brunner, Frau Dr. Brigitte Blöchl-Daum, Herr Martin Bauer, Frau Brigitte Litschauer

ACHTUNG: Unter Berücksichtigung der "ICH-Guideline for Good Clinical Practice" gilt dieser Beschluss **ein Jahr ab Datum der Ausstellung**. Gegebenenfalls hat der Antragsteller eine Verlängerung der Gültigkeit rechtzeitig zu beantragen.

Dieses Votum ist für berechtigte Benutzer/innen in digitaler Form unter der Adresse https://ekmeduniwien.at/vote/10351/download/ abrufbar.

SIGNATURINFORMATIONEN	**Unterzeichner**	Dr. Jürgen Zezula
	Datum/Zeit-UTC	2016-12-01T13:08:33Z
	Prüfinformation	Informationen zur Prüfung der elektronischen Signatur finden Sie unter: https://www.signaturpruefung.gv.at

CHAPTER III – MANUSCRIPT II

EVALUATION OF THE *IN VITRO* ACTIVITY OF ATx201 AND DERIVATIVE COMPOUNDS AGAINST MULTIDRUG RESISTANT BACTERIA

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Abstract

Antibiotic options for the treatment of multidrug resistant bacteria are becoming scarce, highlighting the need to develop novel and effective therapies. One strategy to speed up the antibiotic drug discovery process, is to repurpose existing drugs outside of their original scope.

In this context, niclosamide (ATx201) represents a group of compounds with renewed interest due to displaying a broad range of biological activities, including antimicrobial activity.

In this study we evaluate the antimicrobial potential of 673 of ATx201 derivative compounds, against a broad panel of bacteria, including ESKAPE pathogens. 135 compounds were identified as active towards multiple bacteria. Among the identified hits, ATx13050 was recognized as a promising compound, due to displayed antimicrobial activity and low toxicity. Furthermore, compounds potentiation against Gram-negative bacteria, through drug combinations, it is also described.

Overall, identified derivative compounds, upon further optimization, could represent a potential solution for the treatment of infectious diseases caused by multidrug resistant bacteria.

Keywords: drug repurposing, ATx201, derivative compounds, antibacterial activity, compound potentiation.

Introduction

The spread of antibiotic resistance offers an exceptional challenge to modern medicine. A recent report by the Interagency Coordination Group (IAGG) on Antimicrobial Resistance conveys that, every year, diseases caused by multidrug resistant bacteria cause as many as 700,000 deaths globally and that increasing levels of resistance towards antibiotics continue to be reported worldwide, highlighting the need for more effective therapeutic options [1].

Of special concern are infectious diseases caused by the ESKAPE pathogens. The term ESKAPE, comprises six pathogens that exhibit multidrug resistance through different mechanisms and are able to produce multiple virulence factors. These are *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp. As well, the group is considered the leading cause of nosocomial infections [2,3]. Given this scenario, where antibiotics used in the clinical practice are losing effectiveness, there is an increasing need to focus on the development of novel therapies [4,5]. Conversely, the antibiotic development pipeline faces a discovery void, potentiated by the lack of investment in discovery programs by pharmaceutical companies, due to high costs and low success rates, leading very few potential antibiotics to reach the development stage [5,6].

In response, exploring the potential of existing approved drugs outside of their original indication, become an attractive alternative [7], since information about pharmacological traits (e.g. pharmacokinetics and safety at different development stages) are already described, the time and costs associated with de novo drug discovery programmes may decrease [8,9].

In this context, a drug that has sparked interest is niclosamide. Niclosamide, consists of a clinical approved drug, commercialized as an oral antihelmintic for the treatment of parasitic infections in humans [10]. Additionally, it belongs to a large group of salicylanilides compounds [11,12] and recently, several studies described niclosamide broad range of biological activities and its potential for different clinical applications, including as an antimicrobial agent [13–24].

Studies focusing on salicylanilides antibacterial activity, described niclosamide to be active, in particular, towards Gram-positive, including *S. aureus* and *Staphylococcus epidermidis* [13], *E. faecium* [18] and *Clostridium difficile* [17]. Besides *H. pylori*, niclosamide is described to lack activity towards Gram-negative bacteria (e.g. *Escherichia. coli*) [13,19], which might be related with intrinsic resistance factors such as the presence of efflux pumps [25,26]. Despite the lack of antimicrobial activity by itself, niclosamide was described to potentiate colistin activity against multidrug resistant *A. baumannii* and *K. pneumoniae* [27] and to interfere with *P. aeruginosa* quorum-sensing regulatory system, leading to reduced virulence potential [28].

Nevertheless, different approaches to improve niclosamide pharmacological traits and increase antimicrobial potency can be taken into consideration, including the synthesis of optimized derivative compounds. In fact, different studies focusing on the synthesis of derivative salicylanilides compounds already described promising derivatives with reduced toxicity and improved solubility, while maintaining or improving activity towards multiple pathogens [29–34].

Recently, UNION Therapeutics A/S also described ATx201 (niclosamide) as a potent decolonization against *S. aureus*, including methicillin-resistant *S. aureus* (MRSA) isolates, displaying antibacterial activity at concentrations between 0.125 and 0.5 µg/mL [35]. This finding made ATx201 the lead compound of a promising novel chemical class of derivative compounds, that are being synthesized and studied at UNION.

In this study, we report the antimicrobial potential and spectrum of activity of multiple ATx201 derivative compounds towards different pathogens and the improvement of other pharmacological traits (e.g. toxicity). As well we describe the potential of drug combinations in improving ATx201 activity, in particular towards Gram-negative bacteria.

Materials and Methods

Study design

In this study, we evaluated the spectrum of activity of newly synthesized ATx201 derivative compounds. Compounds antimicrobial potential was assessed in a panel of representative bacterial isolates, including both Gram-positive and Gram-negative bacteria, followed by confirmation of activity in a larger panel of bacteria isolates, covering different levels of resistance. For promising compounds, other pharmacological traits were assessed *in vitro*, such as toxicity and hemolytic potential.

In parallel, ATx201 activity was explored in *E. coli* strains lacking main components of acrAB-TolC efflux pump in order to understand if efflux is contributing to lack of activity observed and how exposure to the compound could affect bacterial growth. As well, drug combinations were studied to improve compounds activity towards Gram-negative bacteria.

Compound information

UNION worked with different chemists to establish a library of compounds designed based on the knowledge acquired about ATx201 activity and microbiological properties. Compounds were designed based on ATx201 chemical structure or on the structures of other related derivatives found to be active during the screenings, accordingly chemical groups were modified or substituted in order to improve chemical potency and other desirable pharmacological traits (e.g. solubility, toxicity). Furthermore, designed structures were submitted to SciFinder® to access novelty of the compounds.

To date, UNION chemical platform comprises 673 compounds, with different chemical scaffolds, that can be divided in four series: ATx10000 (n = 18), ATx11000 (n = 466), ATx12000 (n = 16) and ATx13000 (n = 173). With exception to series ATx12000, which includes compounds

commercially available used as controls, all other series contain newly synthesized compounds that are designed by or on behalf of UNION, by Enamine or collaborators at Wien University.
For screening purposes compounds were dissolved at 10 mg/mL in Dimethyl sulfoxide (DMSO) and stored at -20°C until further use.

Bacterial isolates and reagents

Bacterial isolates included in the different stages of this study were selected from UNION bacterial collection or provided by collaborators. *E. coli* BW25113 and individual gene deletion strains, isolates JW0452-3 (ΔacrA), JW0451-2 (ΔacrB) and JW5503-1 (ΔtolC), were obtained from the Keio collection [36] and isolate MG1655-ΔtolC, bearing a tolC gene deletion, was kindly provided by Professor Ian Henderson (Birmingham University, UK).
Both Gram-positive and Gram-negative bacterial isolates, including clinical isolates with different levels of resistance towards antibiotics used in the clinic were selected, including multidrug resistant strains. Isolates available information can be found in the supplementary material section (Table S1).
All isolates, with exception to *H. pylori* isolates, were preserved in Luria Bertani (LB) broth supplemented with glycerol 15% (v/v) at -80 ºC and cultivated aerobically at 37 ºC in LB agar or tryptic soy agar supplemented with 5% calf blood as appropriate. *H. pylori* isolates were cultured in Columbia agar supplemented with 5% lacked horse blood (Thermo Scientific Oxoid SR0050C), and incubated at 37ºC with 5% CO2 for 72h. Preservation media also differed from the one described above, and the *H. pylori* isolates were preserved in Tryptic Soy broth (TSB) supplemented with 10% fetal bovine serum (FBS) and glycerol 15% (v/v).
All medium and supplements used were purchased from Sigma-Aldrich®.

Antimicrobial potential testing

To determine antimicrobial potential, compounds were screened at a single concentration of 250 µg/mL against a panel of representative strains. Initial bacterial panel included *E. coli* WT 6252,

P. aeruginosa WT 76610, vancomycin resistant-enterococci (VRE) V5 and methicillin- resistant *S. aureus* (MRSA 49). The following *E. coli* strains, JW0452-3 (ΔacrA), JW0451-3 (ΔacrB) and JW5503-1 (ΔtolC), were also included in the initial panel.

The assay was carried out in 96-well plates and in duplicate, following the Clinical and Laboratory Standards Institute (CLSI) guidelines for antimicrobial testing [37].

To access compound activity, optical density at a wavelength of 600 nm was measured and inhibition was calculated as Inhibition (%) = 1 – ($OD_{compound} - OD_{negative\ control}$) / ($OD_{positive\ control} - OD_{negative\ control}$). Compounds with a percentage of inhibition equal or higher 90 [38] were reported as possible hits and screened in a broader panel of bacteria to confirm activity.

In vitro susceptibility screening

Minimum inhibitory concentration (MIC), defined as the lowest concentration of antimicrobial compound needed to inhibit visible growth, was determined using the microbroth dilution method in accordance to the guidelines established by the Clinical and Laboratory Standards Institute (CLSI) [37]. Compounds identified as potential hits were screened, in duplicate, against bacterial isolates described in Table 1 depending on the activity displayed in the previous screening, at the concentration range between 0.125 and 250 μg/mL.

Clindamycin, vancomycin, ciprofloxacin and clarithromycin were used as reference antibiotics as appropriate and, ATx201 was included as control. Drug free, sterility and controls consisting of Mueller-Hinton cation adjusted media, used in the assay, with DMSO were included in each plate. ATCC strains were included as quality controls.

MIC Endpoint was read as the lowest concentration of antimicrobial compound that inhibits growth, as detected by the unaided eye. Also, optical density at a wavelength of 600 nm was measured. Inhibition (%) was calculated as before and MIC values were determined as the minimum concentration with a percentage of inhibition equal or higher than 90% [38].

Additionally, breadth of effect of confirmed hit ATx13050 was assessed against a broader panel of clinical isolates of the species considered of interest, and ATx201 included as control.

Table 1. List of bacterial strains included in the susceptibility screening.

Gram-positive panel	Gram-negative panel	
VRE V5	*E. coli* WT 6252;	*P. aeruginosa* WT 76610
MRSA 49	*E. coli* R10709,	*P. aeruginosa* 9328
S. epidermidis ATCC 12228	*E. coli* KBM16;	*P. aeruginosa* GW1
	E. coli JW0452-3 (ΔacrA),	*K. pneumoniae* WT 7725
	E. coli JW0451-2 (ΔacrB),	*K. pneumoniae* MONT
	E. coli JW5503-1 (ΔtolC)	*K. pneumoniae* 2303
	A. baumannii T23	*H. pylori* ATCC 43504

In vitro cytotoxicity assay

Cytotoxicity of ATx201 and selected derivative compounds was measured in HepG2 cells (ECACC 85011430, Sigma-Aldrich®) as described by Gooyit et al [18]. Cells were grown in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids (NAA) and 1% glutamine and maintained at 37°C and 5% CO2. Briefly, cells were cultured into 96-well plates and incubated for 72h, after that time cells were confirmed to be attached to the bottom of the wells and the medium removed. Subsequently, cells were treated with the test compounds, in duplicate, at concentrations between 0.5 and 125 µg/mL and incubated for 24h. Cell viability was measured by adding 10 µl of resazurin solution (Alamar Blue®) for 3h at 37°C. At the end of the incubation time, fluorescence (FU) was measured at excitation 485 nm and emission at 528 nm. Finally, cell viability was calculated as Cell viability (%) = $(FU_{sample} - FU_{toxicity\ control}) / (FU_{non\text{-}toxicity\ control} - FU_{toxicity\ control})$.

As controls, cells were exposed to DMSO (toxicity control) and complete medium (non-toxic control).

With exception to Alamar Blue, that was purchased from ThermoScientific®, all media and supplements were purchased from Sigma-Aldrich®.

Hemolysis assay

Ability of ATx201 and derivative compounds to cause hemolysis of sheep erythrocytes (tebu-bio®) was determined by adapting the protocols described by Rajamuthiah et al [13] and Eschbach et al [39]. Briefly, in a 96 well plate, 100 µl of 2% sheep erythrocytes suspended in 1xPBS (phosphate buffered saline solution) and added to 100 µl previously serially diluted compounds in 1xPBS, in a final volume of 200 µl per well. Plates were incubated at 37°C with shaking for 90 minutes. After the incubation time, plates were centrifuged at 9000 rpm for 5 minutes and 100 µl of the supernatant from each well of the assay plate were transferred to a new plate and absorbance measured at 540 nm.

Compounds were tested in duplicate, in concentrations between 0.5 and 125 µg/mL. Following controls were included in each assay plate, 2% sheep erythrocytes suspended in 1%Triton X-100 used as hemolysis control and 2% sheep erythrocytes suspended in 1xPBS non-hemolysis control. Compound hemolytic activity, defined as percentage of disrupted sheep erythrocytes at a given compound concentration, was calculated as Hemolysis (%) = ($OD_{sample} - OD_{non\text{-}hemolysis\ control}$) / ($OD_{hemolysis\ control} - OD_{non\text{-}hemolysis\ control}$) [40]. To account for assay variability, a given compound was considered to be non-hemolytic at the higher concentration where hemolysis (%) was below or equal to 10%.

***In vitro* growth analysis and ATx201 dose-response curve in Gram-negatives**

To investigate ATx201 effect towards Gram-negative bacteria, in particular *E. coli*, dose-response curves were set up, and different *E. coli* strains, including bacterial strains lacking main components of acrAB-TolC efflux pump (Table 2), were exposed to different ATx201 concentrations and growth was followed for 24h.

Briefly, an overnight culture of each bacterial strain was prepared in LB and incubated at 37˚C with shacking. The next day the culture OD was measured, and 0.5 mL of bacterial suspension added to media supplemented with 0, 250, 500, 1000 µg/mL of ATx201. OD at 600 nm was

measured at time 0 and every hour up to time 8. A final measurement was taken after 24h. Biological replicates were included, and the assay carried out at two different time points. Prior to the experiment the minimal inhibitory concentration (MIC) of each bacterial strain included in the assay was determined using the previously described method.

Table 2. List of *E. coli* bacterial strains included in the assay.

Bacterial Isolate ID	Mutation
BW25113	Parental strain
E. coli JW0452-3 (ΔacrA)	Delection of acrA membrane fusion protein
E. coli JW0451-2 (ΔacrB)	Delection of acrB transporter
MG1655-ΔtolC	Deletion of tolC outer membrane factor

Potentiation of ATx201 and derivatives compounds activity through combination with UNIP01

ATx201 and selected derivative compounds were screened, in duplicate, in combination with different concentrations of UNIP01. Briefly, UNIP01 MIC was determined for *E. coli* WT 6252, as described before and found to be 250 μg/mL. After MIC determination, compounds of interest were combined with 0.5xMIC (125 μg/mL), 0.25xMIC (62.5 μg/mL), 0.125xMIC (31.2 μg/mL) and 0.06xMIC (15.6 μg/mL) of UNIP01 and tested between the concentration range of 0.125 to 250 μg/mL.

Compounds included were selected based on activity displayed against *E. coli* WT 6252.

Results and Discussion

Antibacterial activity spectrum of ATx201 derivative compounds

In an initial effort to access the antimicrobial potential of ATx201 derivative compounds, the compounds were tested against a panel of representative strains, including genetic modified *E. coli* strains, lacking genes related with efflux pumps, specifically *acrA, acrB* and *tolC*, involved in the multidrug resistant phenotype displayed by Gram-negative bacteria (e.g. *E. coli*).

As expected, a higher number of active derivative compounds was observed among Gram-positive bacteria, such as MRSA and enterococci. This relates to the activity displayed by ATx201, already described in multiple studies [13,18,35]. Out of 673 compounds, 163 compounds were shown to be active against Gram-positive bacteria, whereas 78 compounds were identified as having activity towards Gram-negative isolates. Upon secondary screening, activity of 113 compounds was established against Gram-positive bacteria and 63 compounds were confirmed to be active at least against one isolate in the Gram-negative panel (Figure 1A).

Overall, 135 different compounds were identified as having different levels of activity against the isolates tested at concentrations up to 250 µg/mL (Figure 1B and 1C). Though activity at 250 µg/mL it is not a desirable concentration to move forward to *in vivo* testing, these compounds can provide useful knowledge about structure and biological activity relation (SAR) and be considered for further optimization.

Compounds belonging to series ATx10000, upon secondary screening, were shown to lack activity against all isolates, whereas series ATx11000 provided several hits with activity between concentrations of 32 and 250 µg/mL, with compound ATx11197 being the most relevant with activity against all bacterial isolates included in the screening panel. Series ATx11000 was not tested against *H. pylori* isolates, however, based on data collected from other series, where compounds lacking activity against other Gram-negatives, were active against *H. pylori*, it could be that possible hits were missed. In the case of series ATx12000 and ATx13000, different hits were identified against isolates tested, at concentrations between 0.125 and 250 µg/mL.

As stated above, series ATx12000 includes commercially available compounds that were used as controls and mainly in comparison with ATx201. In this scenario, ATx12000 and ATx12004 were considered the most relevant compounds in the group since they displayed activity against Gram-negative isolates as well, albeit with lower potency. In the case of Gram-positives, specifically MRSA, neither of the control compounds appeared to be more potent than ATx201, although activity was found to be 0.5 µg/mL for ATx12000, ATx12001 and ATx12004. As well,

compounds UNI12007 and UNI12010 were found to be the most relevant within the series against *H. pylori* with MICs in the same interval range as ATx201.

Furthermore, compounds ATx13004, ATx13006, ATx13050, UNI13106, UNI13107, UNI13114 and UNI13153, belonging to series ATx13000 were identified as the most relevant against enterococci strains (MICs between 0.125 and 2 µg/mL). With exception to ATx13050, UNI13106 and UNI13153, the mentioned compounds seemed to be selective for enterococci. In the case of MRSA activity, the most relevant compounds are ATx13039, ATx13050, UNI13101, UNI13103, UNI13106 and UNI13153. None of the compounds being selective towards the species alone. In the case of *H. pylori*, among the ATx13000 series, a total of 19 compounds were identified to have activity, with the most relevant compounds being ATx13050, UNI13139 and UNI13145, with MICs of 0.125 and 2 µg/mL, respectively.

Overall, the above-mentioned compounds also displayed an increased solubility in DMSO when compared to ATx201.

MIC values observed for control antibiotics included were found to be in accordance with the values described by the CLSI [37] and EUCAST [42] breakpoints for reference strains (supplementary Table S2). MICs for compounds confirmed as hits are described in supplementary Tables S3 and S4. Upon further investigation, screening data regarding *E. coli* Δ*tolC* (JW0452-3) was excluded due to the strain lacking the intended mutation.

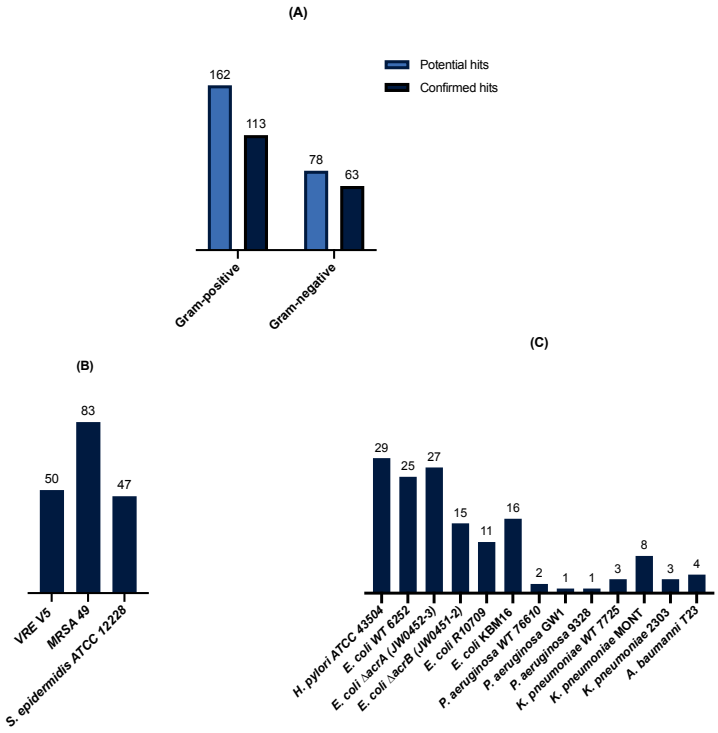

Figure 1. Number of hits identified by bacterial group and number of confirmed active compounds against different bacterial isolates. (A) Describes the number of compounds identified as potential and confirmed hits for Gram-positive and Gram-negative. (**B)** and **(C)** Describes number of active compounds confirmed per isolate, Gram-positive and Gram-negative panels, respectively.

Breadth of effect of compound ATx13050

Compound ATx13050 was identified in the secondary screening as a promising hit against staphylococci, enterococci and *H. pylori* isolates, with MICs between < 0.125 and 1 µg/mL. Due to the displayed activity, the compound was further tested against a broader panel of clinically relevant isolates of the mentioned species, including both methicillin-susceptible and methicillin-resistant *S. aureus* (n = 11), *enterococci* (n = 54) and *H. pylori* (n = 49) strains.

MIC distribution shown to be variable, in particular in the case of *staphylococci* and *enterococci* isolates included, with MICs values ranging from 0.125 to 125 µg/mL (Figure 2A and 2B) As well, data suggests ATx13050 to be less active against *S. aureus* when compared with ATx201 activity in the same set of isolates, while displaying a similar MIC distribution in *enterococci* (Figure 3A and 3B).

In the case of *H. pylori*, similarly to the activity displayed by ATx201, ATx13050 MIC was found to be consistently below 1 µg/mL independently of the clinical isolate screened (Figure 2C and Figure 3C).

While we hypothesized ATx13050 MIC variability to be associated with the different antibiotic resistance profile and genomic background of the screened clinical isolates and possible resistance mechanisms within the different species, further analysis would be necessary to understand the reasoning behind it. Including, screening ATx13050 in a larger set of strains, specifically in the case of *staphylococci* isolates, and, investigate further the compound possible mode of action and biological target and if it differs from the one displayed by ATx201.

Both ATx13050 and ATx201 screening data can be found in supplementary Table S5.

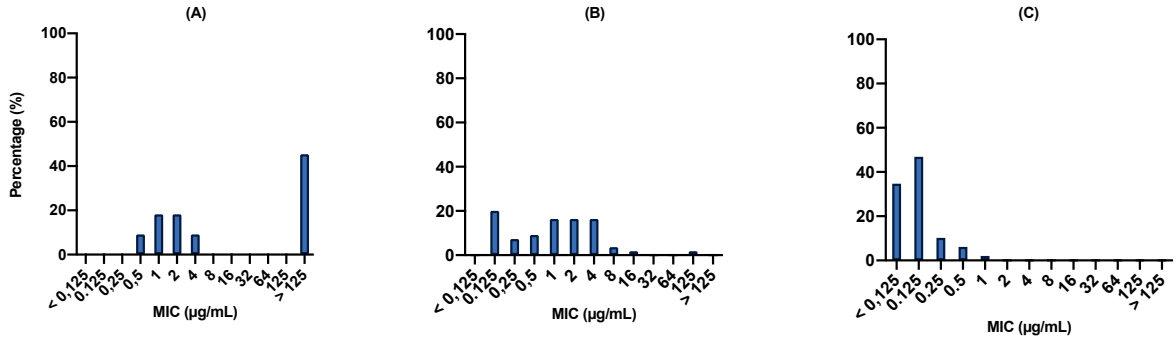

Figure 2. ATx13050 MIC distribution (%). (A) *staphylococci* isolates (n = 11); **(B)** *enterococci* isolates (n = 54); **(C)** *H. pylori* isolates (n = 49).

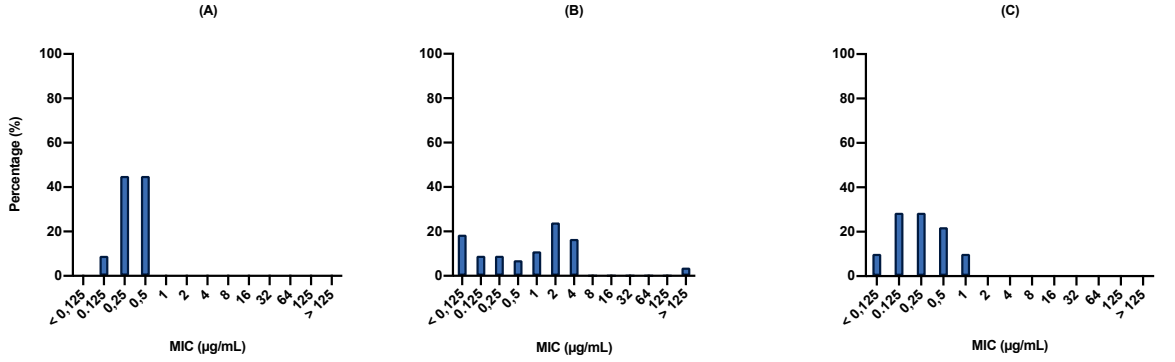

Figure 3. ATx201 MIC distribution (%). (A) *staphylococci* isolates (n = 11); **(B)** *enterococci* isolates (n = 54); **(C)** *H. pylori* isolates (n = 49).

Toxicity and hemolytic activity of ATx201 derivatives

The potential effect of ATx201 and derivative compounds in cell viability was assessed, by exposing HepG2 cells, to different concentrations of the compounds. Data suggests that cell viability decreases in a concentration-dependent manner for all tested compounds (Supplementary Figure S1). Treatment of HepG2 cells with ATx201 showed a marked decrease of viability at concentrations as low as *0.5 μg/mL* (lowest concentration tested). Whereas cell viability seemed to improve, when the cells were exposed to 0.5 μg/mL of derivative compounds. For example, we observed that compound ATx13050 did not have an apparent effect in cell viability at concentrations at least up to 4 μg/mL, which is 2-fold higher than its MIC range against *H. pylori* clinical isolates.

Nevertheless, despite the observed *in vitro* cytotoxicity, towards HepG2 cells, caused by ATx201 and derivative compounds, ATx201 has proven to be a relatively safe drug since it was first commercialized for the treatment of antihelmintic infections in humans [17] and, similar observations were made during the *in vivo* assays and clinical trials conducted by UNION [35].

Furthermore, ability of compounds to cause rupture of sheep erythrocytes was investigated.

With exception of compounds ATx12004, ATx12002 and ATx11013 where hemolysis was observed at concentrations above 32 and 64 μg/mL, respectively, collected data suggests that the tested compounds do not cause hemolysis at concentrations as high as 125 μg/mL, a concentration higher than the one necessary for the compounds to display antibacterial activity (Supplementary Figure S2).

It is important to note that these data do not guarantee compounds safety and that further testing is necessary to trace the compounds safety profile, however it demonstrates derivative compounds potential.

ATx201 activity towards *E. coli*

The role of multidrug efflux pumps as a primary mechanism conferring resistance towards antibiotics in Gram-negative bacteria, in particular the clinically relevant acrAB-TolC efflux pump in *E. coli* is well established [43]. As a way to investigate ATx201 activity towards *E. coli* and the possible role of efflux in the lack of ATx201 activity observed, we investigated the potential effect of individual deletions for components of the acrAB-TolC efflux pump in ATx201 antimicrobial potential, by determining the minimal inhibitory concentration (MIC).

Although a MIC was not established against strains *E. coli* BW25113 WT and deletion mutants *E. coli* JW0452-3 (ΔacrA) and *E. coli* JW0451-2 (ΔacrB) (MIC > 250 µg/mL), it was possible to establish a MIC for strain MG1655-ΔtolC (MIC = 1 µg/mL). The fact that other deletions did not had an effect in the MIC, suggests that tolC (an outer membrane channel) and tolC-mediated efflux could play an important role in expelling ATx201 from the bacterial cell. Other studies already reported that deletion of the tolC contributed to increase antibiotic susceptibility towards Gram-negative bacteria [44,45] and recently similar results were described by Copp et al for niclosamide [25,46].

To further investigate ATx201 lack of activity observed towards both the parental strain and deletion mutants, the above-mentioned strains were exposed to different concentrations of ATx201 for 24h.

Our data suggests that by exposing the different *E. coli* strains, in specific strains *E. coli* BW25113 WT (Figure 4A) and deletion mutants *E. coli* JW0452-3 (ΔacrA) (Figure 4B) and *E. coli* JW0451-2 (ΔacrB) (Figure 4C), to 250 µg/mL of ATx201, the lag phase, although at a very small extension, seems to be longer and consequently the exponential growth phase is delayed in comparison to the no drug control, however stationary phase seems to be reached around the same time point and with a similar bacterial density. The observed delay could be a consequence of *E. coli* adapting to the supplemented media rather than a direct effect of ATx201 in bacterial growth rate. Similar findings regarding *E. coli* strains media adaption were reported elsewhere [47].

Nevertheless, when exposing strain MG1655-ΔtolC to 250 µg/mL of ATx201, growth is arrested (Figure 4D), which is in accordance with the MIC obtained. This also further corroborates the contribution of tolC towards ATx201 lack of activity.

Finally, concentrations 500 and 1000 µg/mL were excluded due to compound precipitation during the experiment.

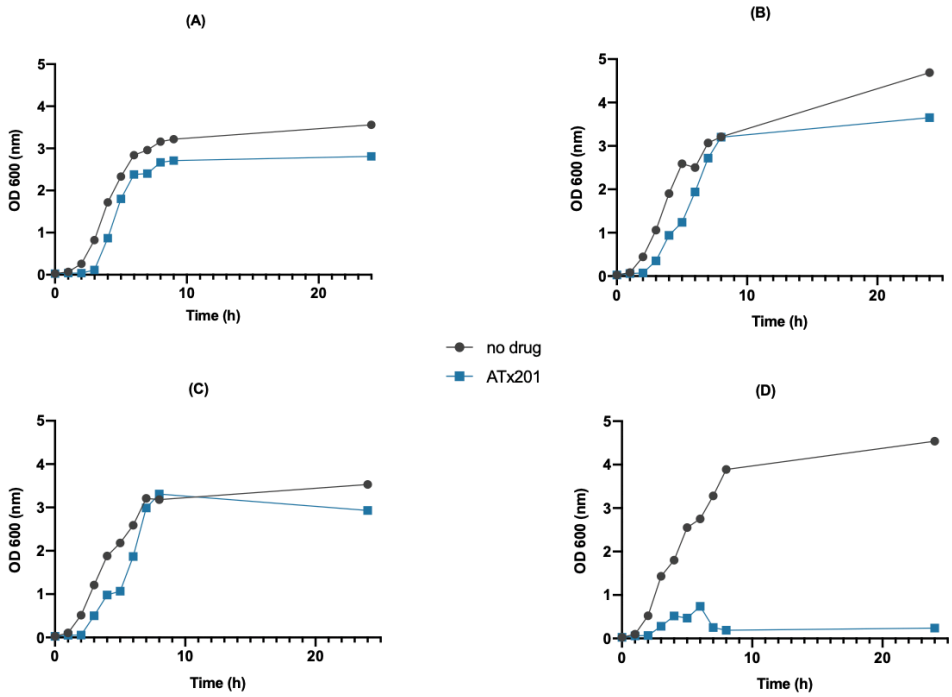

Figure 4. Growth-rates of *E. coli* strains exposed to 250 µg/ml of ATx201. (A) *E. coli* BW25113; **(B)** *E. coli* JW0452-3 (ΔacrA) **(C)** *E. coli* JW0451-2 (ΔacrB) **(D)** *E. coli* MG1655-ΔtolC. ATx201 was shown to impair and affect the growth the *E. coli* strain lacking the tolC gene at a higher extent, suggesting tolC involvement in ATx201 efflux.

UNIP01 potentiates ATx201 and derivatives antibacterial effect in *E. coli*

The use of combined antibacterial therapies can contribute to decrease the emergence of spontaneous resistance, as well to re-established or confer efficacy to antimicrobial compounds, where activity was not observed before [28,48]. Due to ATx201 activity recorded towards MG1655-ΔtolC, it was hypothesized that ATx201 activity could be potentiated towards Gram-negative

bacteria via drug combinations. We selected UNIP01[(1)], a commercially available therapeutic agent, due to its ability to sensitize Gram-negative bacteria (e.g. *E. coli*) to the action of antibiotics only active towards Gram-positive bacteria. Although, the compound mode of action in this context, is not very well known, it is believed to potentiate antibiotic action by disrupting the outer membrane of Gram-negative bacteria through interacting with membrane lipopolysaccharides (LPS).

We investigated the activity of ATx201 and selected derivatives in combination with different concentrations below UNIP01 MIC against *E. coli* WT 6252. Although, ATx201 alone did not have antimicrobial activity against the tested strain (MIC > 250 µg/mL), when combined with 125 µg/mL of UNIP01, ATx201 activity was potentiated and the MIC determined to be 8 µg/mL. Similar observations were made for ATx12001 and ATx12004. No potentiation was observed for compounds ATx13023, ATx13049 and ATx13056 (Table 3).

Changes in activity were not observed when screening the compounds together with lower concentrations of UNIP01 (0.25xMIC (62.5 µg/mL), 0.125xMIC (31.2 µg/mL) and 0.06xMIC (15.6 µg/mL)).

While ATx201 and derivative compounds are not particularly active against Gram-negative bacteria, with exception to *H. pylori*, our data suggests that compounds activity might be potentiated through combination with commercially available compounds, such as UNIP01, and in this way increase compounds activity spectrum. Multiple studies describe compounds potentiation against resistant bacteria through drug combination [27,40,49,50]. As well, Copp et al described niclosamide potentiation against *E. coli* through combination with phenylalanine-arginine ß-naphthylamide (PAßN) [25,46].

[(1)] Name was changed due to IP interests. As well, literature refering to the compound was not included.

Table 3. ATx201 and derivatives alone and in combination with UNIP01.

Isolate	Compound	MIC alone (μg/mL)	MIC in combination with UNIP01 (125 μg/mL)
***E. coli* WT 6252**	ATx201	-	8
	ATx12001	250	8
	ATx12004	64	2
	ATx13023	250	250
	ATx13049	250	250
	ATx13056	250	250
	UNIP01	250	-

Conclusions

The current antibiotic resistance crisis imposes the need to develop novel antibiotic options, in particular for the treatment of bacterial infections caused by multidrug resistant Gram-negative pathogens, where options are becoming worryingly scarce [49].

In this study, we investigated the antimicrobial potential of ATx201 and ATx201 derivative compounds against clinically relevant pathogens. We identified 113 compounds to be active against Gram-positive bacteria, although neither of the derivative compounds displayed a higher activity when compared to ATx201. As well, 63 compounds were identified as active towards Gram-negative pathogens, albeit at higher concentrations (32 to 250 μg/mL). The exception was *H. pylori* were compounds were identified to be active at concentrations as low as 0.125 μg/mL. Although, out of 673 compounds only 135 different compounds were found to be active and mostly at concentrations that are not considered a set of desirable concentrations, when looking for a potential antimicrobial compound, these compounds can provide valuable information, about structure and biological activity, contributing to increase knowledge about the class and contribute to compound optimization. Additionally, among the derivative compounds, we identified ATx13050 as a promising hit, as it shown activity against multiple species and displayed a consistent MIC (< 1 μg/mL) against *H. pylori*. Moreover, it demonstrated a lower cellular toxicity towards HepG2 cells. Based on our findings, compounds ATx13050 warrants further investigation as a potential novel antibacterial for the treatment of multidrug resistant bacteria, in particular those caused by *H. pylori.*

Furthermore, our data suggests that tolC-mediated efflux might play a major role in the lack of activity displayed by ATx201 towards Gram-negative bacteria. This was further corroborated by ATx201 potentiation through drug combination with a compound involved in membrane sensitization. Even though the "optimal drug combination" does not exist since it is always dependable on the strain susceptibility background, drug combinations represent an attractive alternative to combat antibiotic resistance [27] and by following this approach ATx201 and derivative could potentially become effective against Gram-negative bacteria.

Taken together, our data suggests that with continuous optimization, ATx201 and ATx201 derivative compounds could represent a novel class of antimicrobial compounds with broad applicability and superior activity when compared with available clinical options.

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Professor Thomas Erker for providing ATx11000 series compounds. Professor Ian Handerson, for kindly share the *E. coli* MG1655 Δ*tolC* strain. And, Professor Leif Andersen and Janetta top for providing the *H. pylori* and enterococci clinical isolates, respectively, included in this study.

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Supplementary Material

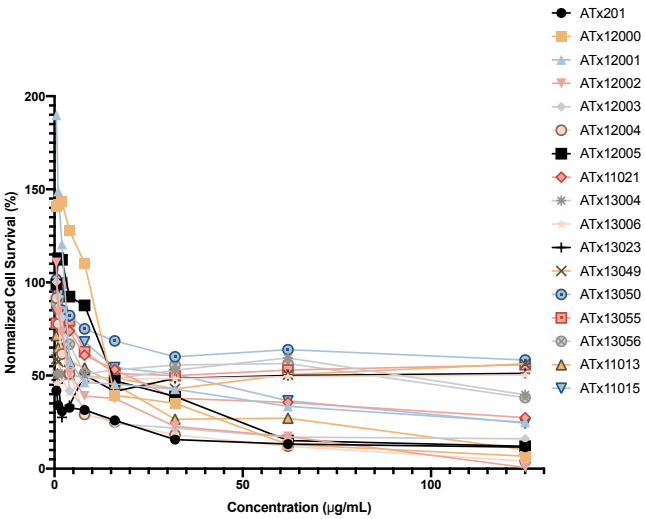
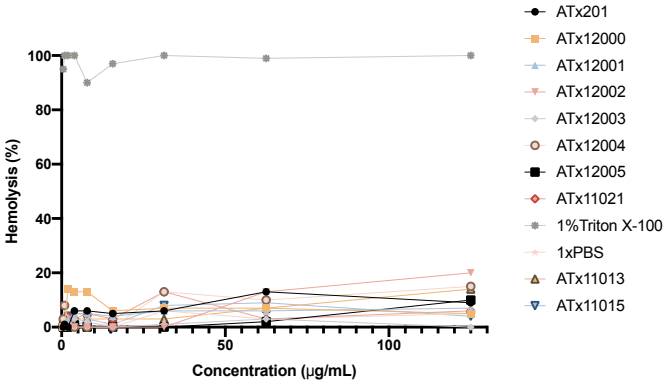


Figure S1. *In vitro* toxicity towards HepG2 cells. Cell viability was evaluated after treatment with two-fold serially diluted concentrations (0.5 to 125 µg/mL) of selected compounds, using the Alamar blue assay.



Compound ID	Non-hemolytic concentration (µg/mL)
ATx201	125
ATx12000	125
ATx12001	125
ATx12002	32
ATx12003	125
ATx12004	64
ATx12005	125
ATx11013	64
ATx11015	125
ATx11021	125

Figure S2. *In vitro* hemolytic activity of selected compounds assessed in red blood cells. Hemolysis was evaluated after treatment with two-fold serially diluted concentrations (0.5 to 125 µg/mL). Compounds were considered to be non-hemolytic at the higher concentration displaying a hemolysis (%) below or equal to 10%.

Table S1. Bacterial isolates included in this study. n.d.: not determined.

Isolates ID	Relevant Information	MLST	Origin
E. coli			
Wild-Type 6252	No information available regarding resistance profile	n.d.	Clinical isolate
R10709	Extended spectrum beta-lactamase (ESBL)	n.d.	Clinical isolate
KBM16	Class A carbapenemase producer	n.d.	Clinical isolate
BW25113	Parental strain	n.d.	Keio collection
JW0452-3 (ΔacrA)	Delection of acrA membrane fusion protein	n.d.	Keio collection
JW0451-2 (ΔacrB)	Delection of acrB transporter	n.d.	Keio collection
JW5503-1 (ΔtolC)	Deletion of tolC outer membrane factor	n.d.	Keio collection
MG1655-ΔtolC	Deletion of tolC outer membrane factor	n.d.	Provided by Professor Ian Henderson
ATCC 25922	CLSI reference strain	n.d.	ATCC
P. aeruginosa			
Wild type 76610	No information available regarding resistance profile	n.d.	Clinical isolate
9328	Extended spectrum beta-lactamase (ESBL)	n.d.	Clinical isolate
GW1	Class A carbapenemase producer	n.d.	Clinical isolate
ATCC 27853	CLSI reference strain	n.d.	ATCC
K. pneumoniae			
Wild type 7725	No information available regarding resistance profile	n.d.	Clinical isolate
MONT	Extended spectrum beta-lactamase (ESBL)	n.d.	Clinical isolate
2303	Class A carbapenemase producer	n.d.	Clinical isolate
A. baumannii			
T23	Extended spectrum beta-lactamase (ESBL)	n.d.	Clinical isolate
S*taphylococci*			
S. epidermidis ATCC 12228	CLSI reference strain	n.d.	ATCC
S. aureus ATCC 29213	CLSI reference strain	n.d.	ATCC
S. aureus Newman		ST8	Laboratory strain
MRSA 02	Community-acquired MRSA. South East Pacific clone	ST30	Clinical isolate
MRSA 03	Community-acquired MRSA. Clone USA400. PVL positive.	ST1	Clinical isolate
MRSA 10	Epidemic MRSA	ST22	Clinical isolate
MRSA 49	Hospital-acquired MRSA	ST125	Clinical isolate
MRSA 98	Hospital-acquired MRSA	ST188	Clinical isolate
MRSA 103	Hospital-acquired MRSA	ST149	Clinical isolate

S. aureus K00086	Methicillin-sensitive *S. aureus* (MSSA)	n.d.	Clinical isolate
S. aureus K000796	Methicillin-sensitive *S. aureus* (MSSA)	n.d.	Clinical isolate
S. aureus mupA 13	Methicillin-sensitive *S. aureus* (MSSA)	n.d.	Clinical isolate
Enterococci			
E. faecallis ATCC 29212	CLSI reference strain	n.d.	ATCC
VRE 5	Vancomycin-resistant enterococci	n.d.	Clinical isolate
VRE V34	Vancomycin-resistant enterococci	n.d.	Clinical isolate
VRE V42	Vancomycin-resistant enterococci	n.d.	Clinical isolate
VRE V55	Vancomycin-resistant enterococci	n.d.	Clinical isolate
VRE V59	Vancomycin-resistant enterococci	n.d.	Clinical isolate
VRE V77	Vancomycin-resistant enterococci	n.d.	Clinical isolate
VRE V95	Vancomycin-resistant enterococci	n.d.	Clinical isolate
VRE V140	Vancomycin-resistant enterococci	n.d.	Clinical isolate
VRE V171	Vancomycin-resistant enterococci	n.d.	Clinical isolate
VRE V181	Vancomycin-resistant enterococci	n.d.	Clinical isolate
E. faecium E084	Presence of vancomycin/teicoplanin A-type resistance protein vanA	ST5	Clinical isolate
E. faecium E088	Presence of vancomycin/teicoplanin A-type resistance protein vanA	ST25	Clinical isolate
E. faecium E097	Vancomycin resistance genes not detected	ST92	Clinical isolate
E. faecium E0100	Vancomycin resistance genes not detected	ST1123	Clinical isolate
E. faecium E0157	No information available regarding vancomycin resistance	n.d.	Clinical isolate
E. faecium E0745	Presence of vancomycin/teicoplanin A-type resistance protein vanA	ST16	Clinical isolate
E. faecium E0804	No information available regarding vancomycin resistance	n.d.	Clinical isolate
E. faecium E0810	No information available regarding vancomycin resistance	n.d.	Clinical isolate
E. faecium E1071	Presence of vancomycin/teicoplanin A-type resistance protein vanA	ST32	Clinical isolate
E. faecium E1162	Vancomycin resistance genes not detected	ST17	Clinical isolate
E. faecium E1583	Vancomycin resistance genes not detected	ST161	Clinical isolate
E. faecium E1716	Vancomycin resistance genes not detected	ST17	Clinical isolate
E. faecium E1717	Vancomycin resistance genes not detected	ST18	Clinical isolate
E. faecium E1721	Vancomycin resistance genes not detected	ST169	Clinical isolate
E. faecium E1764	Presence of vancomycin/teicoplanin A-type resistance protein vanA	ST6	Clinical isolate
E. faecium E1780	Vancomycin resistance genes not detected	ST144	Veterinary isolate
E. faecium E4244	Presence of vancomycin/teicoplanin A-type resistance protein vanA	ST310	Veterinary isolate
E. faecium E4344	Vancomycin resistance genes not detected	ST623	Clinical isolate

E. faecium E4425	Vancomycin resistance genes not detected	ST266	Veterinary isolate
E. faecium E4426	Vancomycin resistance genes not detected	ST19	Veterinary isolate
E. faecium E6009	Presence of vancomycin/teicoplanin A-type resistance protein vanA	ST547	Clinical isolate
E. faecium E6035	Presence of vancomycin/teicoplanin A-type resistance protein vanA	ST17	Clinical isolate
E. faecium E6036	Presence of vancomycin/teicoplanin A-type resistance protein vanA	ST78	Clinical isolate
E. faecium E7170	Presence of vancomycin/teicoplanin A-type resistance protein vanA	ST440	Clinical isolate
E. faecium E7192	Presence of vancomycin/teicoplanin A-type resistance protein vanA	ST78	Clinical isolate
E. faecium E7333	Presence of vancomycin/teicoplanin A-type resistance protein vanA	ST290	Clinical isolate
E. faecium E7424	Presence of vancomycin B-type resistance protein vanB	ST78	Clinical isolate
E. faecium E7641	Presence of vancomycin/teicoplanin A-type resistance protein vanA	ST117	Clinical isolate
E. faecium E7842	Presence of vancomycin/teicoplanin A-type resistance protein vanA	ST730	Clinical isolate
E. faecium E7874	Presence of vancomycin B-type resistance protein vanB	ST192	Clinical isolate
E. faecium E7876	Presence of vancomycin/teicoplanin A-type resistance protein vanA	ST203	Clinical isolate
E. faecium E8028	Presence of vancomycin/teicoplanin A-type resistance protein vanA	ST203	Clinical isolate
E. faecium E8042	Presence of vancomycin/teicoplanin A-type resistance protein vanA	ST18	Clinical isolate
E. faecium E8042	Presence of vancomycin/teicoplanin A-type resistance protein vanA	ST18	Clinical isolate
E. faecium E8043	Presence of vancomycin D-type resistance protein vanD	ST203	Clinical isolate
E. faecium E8202	Presence of vancomycin/teicoplanin A-type resistance protein vanA	ST117	Clinical isolate
E. faecium E8436	Presence of vancomycin/teicoplanin A-type resistance protein vanA and of vancomycin B-type resistance protein vanB	ST552	Clinical isolate
E. faecium E8437	Presence of vancomycin/teicoplanin A-type resistance protein vanA	ST80	Clinical isolate
E. faecium E8444	Presence of vancomycin/teicoplanin A-type resistance protein vanA	ST80	Clinical isolate
E. faecium E8460	Presence of vancomycin B-type resistance protein vanB	ST117	Clinical isolate
E. faecium E8671	Vancomycin resistance genes not detected	ST39	Clinical isolate

E. faecium E9004	Presence of vancomycin B-type resistance protein vanB	ST117	Clinical isolate
E. faecium E9006	Presence of vancomycin/teicoplanin A-type resistance protein vanA	ST80	Clinical isolate
E. faecium E9029	No information regarding resistance genes available	n.d.	Clinical isolate
H. pylori			
ATCC 43504	Quality control CLSI suggested strain for susceptibility testing	n.d.	ATCC
008A	No information available regarding resistance profile	n.d.	Clinical isolate
013A	No information available regarding resistance profile	n.d.	Clinical isolate
020A	No information available regarding resistance profile	n.d.	Clinical isolate
024K	No information available regarding resistance profile	n.d.	Clinical isolate
025A	No information available regarding resistance profile	n.d.	Clinical isolate
028K	No information available regarding resistance profile	n.d.	Clinical isolate
030A	No information available regarding resistance profile	n.d.	Clinical isolate
046A	No information available regarding resistance profile	n.d.	Clinical isolate
055A	No information available regarding resistance profile	n.d.	Clinical isolate
071k	No information available regarding resistance profile	n.d.	Clinical isolate
073k	No information available regarding resistance profile	n.d.	Clinical isolate
084K	No information available regarding resistance profile	n.d.	Clinical isolate
091k	No information available regarding resistance profile	n.d.	Clinical isolate
102k	No information available regarding resistance profile	n.d.	Clinical isolate
108A	No information available regarding resistance profile	n.d.	Clinical isolate
114k	No information available regarding resistance profile	n.d.	Clinical isolate
130k	No information available regarding resistance profile	n.d.	Clinical isolate
1353	No information available regarding resistance profile	n.d.	Clinical isolate
1427	No information available regarding resistance profile	n.d.	Clinical isolate
1436	No information available regarding resistance profile	n.d.	Clinical isolate
1494	No information available regarding resistance profile	n.d.	Clinical isolate
2237	No information available regarding resistance profile	n.d.	Clinical isolate
2238	No information available regarding resistance profile	n.d.	Clinical isolate
2278	No information available regarding resistance profile	n.d.	Clinical isolate
2390	No information available regarding resistance profile	n.d.	Clinical isolate
2507	No information available regarding resistance profile	n.d.	Clinical isolate
2581	No information available regarding resistance profile	n.d.	Clinical isolate
2589	No information available regarding resistance profile	n.d.	Clinical isolate
25A	No information available regarding resistance profile	n.d.	Clinical isolate
2797	No information available regarding resistance profile	n.d.	Clinical isolate
27A	No information available regarding resistance profile	n.d.	Clinical isolate
3127	No information available regarding resistance profile	n.d.	Clinical isolate
3175	No information available regarding resistance profile	n.d.	Clinical isolate
3207	No information available regarding resistance profile	n.d.	Clinical isolate

3250	No information available regarding resistance profile	n.d.	Clinical isolate
3748	No information available regarding resistance profile	n.d.	Clinical isolate
379935-3	No information available regarding resistance profile	n.d.	Clinical isolate
467	No information available regarding resistance profile	n.d.	Clinical isolate
573	No information available regarding resistance profile	n.d.	Clinical isolate
585	No information available regarding resistance profile	n.d.	Clinical isolate
74A	No information available regarding resistance profile	n.d.	Clinical isolate
76k	No information available regarding resistance profile	n.d.	Clinical isolate
906	No information available regarding resistance profile	n.d.	Clinical isolate
95k	No information available regarding resistance profile	n.d.	Clinical isolate
99A	No information available regarding resistance profile	n.d.	Clinical isolate
AK043	No information available regarding resistance profile	n.d.	Clinical isolate
GU009K	No information available regarding resistance profile	n.d.	Clinical isolate
GU010A	No information available regarding resistance profile	n.d.	Clinical isolate

Table S2. Quality control strains and respective MIC, according to CLSI guidelines, included in the screening assays. NT: antibiotic was not tested in the bacterial isolate.

	MIC (µg/mL)			
Strains ID	**Clindamycin**	**Vancomycin**	**Ciprofloxacin**	**Clarithromycin**
S. aureus ATCC 29213	0.125	1	NT	NT
E. faecalis ATCC 29212	NT	2	NT	NT
E. coli ATCC 25922	NT	NT	0.016	NT
P. aeruginosa ATCC 27853	NT	NT	0.5	NT
H. pylori ATCC 43504	NT	NT	NT	0.125

Table S3. Compounds confirmed as hits in the Gram-positive panel. MIC values ≤ 8 µg/mL (green); between 16 and 32 µg/mL (yellow); ≥ 64 µg/mL (red); > 250: MIC was not determined in the concentration range tested; NT: compound was not tested in the bacterial isolate.

		Bacterial Isolates (MIC µg/mL)		
Compound ID	Molecular weight (g/mol)	VRE V5	*S. epidermidis* ATCC 12228	MRSA 49
ATx201 (niclosamide)	327	>250	0.5	0.125
ATx11015	357	NT	NT	125
ATx11021	424	NT	NT	64
ATx11034	220	NT	250	> 250
ATx11035	245	NT	250	250
ATx11036	296	NT	250	250
ATx11052	231	250	> 250	NT
ATx11054	248	250	250	> 250
ATx11056	278	NT	250	250
ATx11058	259	NT	250	250
ATx11067	310	250	250	32
ATx11071	169	NT	> 250	250
ATx11099	300	NT	250	> 250
ATx11152	363	NT	> 250	250
ATx11170	283	> 250	250	> 250
ATx11171	283	> 250	250	> 250
ATx11173	292	> 250	> 250	250
ATx11174	294	> 250	> 250	250
ATx11181	241	> 250	250	250
ATx11183	257	> 250	> 250	250
ATx11197	200	32	250	32

ATx11217	260	250	> 250	250
ATx11237	216	250	> 250	250
ATx11272	241	> 250	125	64
ATx11287	195	> 250	125	64
ATx11291	251	64	64	64
ATx11304	266	> 250	NT	250
ATx11309	307	64	> 250	250
ATx11310	321	> 250	125	250
ATx11317	246	250	> 250	250
ATx11393	210	250	> 250	> 250
ATx11394	210	250	> 250	> 250
ATx11401	256	250	> 250	250
ATx11402	286	250	250	250
ATx11407	252	250	NT	> 250
ATx11410	352	32	NT	> 250
ATx11433	151	250	NT	> 250
ATx11436	237	64	NT	> 250
ATx11456	249	> 250	NT	250
ATx12000	663	NT	NT	0.5
ATx12001	441	NT	NT	0.5
ATx12002	307	NT	NT	62
ATx12003	137	NT	NT	125
ATx12004	449	NT	NT	0.5
UNI12007	314	NT	NT	1
UNI12009	251	NT	NT	16

UNI12010	847	NT	NT	8
UNI12011	388	NT	4	4
UNI12014	516	NT	NT	0.25
UNI12015	747	NT	NT	16
ATx13004	272	0.5	> 250	250
ATx13006	313	0.25	250	> 250
ATx13007	323	> 250	16	> 250
ATx13008	292	> 250	250	> 250
ATx13009	279	250	250	> 250
ATx13010	287	> 250	250	> 250
ATx13011	290	> 250	16	250
ATx13012	290	> 250	250	125
ATx13015	344	250	125	250
ATx13016	348	> 250	250	250
ATx13017	338	> 250	250	> 250
ATx13019	306	> 250	16	> 250
ATx13027	401	250	8	> 250
ATx13028	338	> 250	> 250	64
ATx13030	325	> 250	> 250	125
ATx13031	335	> 250	> 250	250
ATx13035	335	> 250	> 250	8
ATx13037	307	> 250	1	> 250
ATx13039	358	> 250	125	1
ATx13042	337	> 250	> 250	64
ATx13044	313	> 250	> 250	8

ATx13045	399	> 250	125	250
ATx13049	279	> 250	125	> 250
ATx13050	315	0.125	> 250	1
ATx13051	400	250	250	250
ATx13053	312	> 250	250	> 250
ATx13055	316	64	16	32
ATx13056	361	64	8	16
ATx13057	351	32	64	64
ATx13058	298	> 250	250	> 250
ATx13063	298	> 250	> 250	250
ATx13066	352	> 250	250	250
ATx13068	387	> 250	> 250	125
ATx13069	256	> 250	250	> 250
ATx13073	267	64	250	> 250
ATx13075	485	> 250	> 250	125
ATx13082	326	NT	250	> 250
UNI13097	302	125	> 250	250
UNI13098	289	> 250	> 250	8
UNI13099	262	250	> 250	> 250
UNI13100	274	250	> 250	64
UNI13101	327	> 250	> 250	0.25
UNI13103	326	4	> 250	0.25
UNI13106	296	0.25	> 250	1
UNI13107	314	0.25	> 250	> 250
UNI13111	383	> 250	> 250	125

UNI13113	375	> 250	250	> 250
UNI13114	429	0.25	> 250	64
UNI13120	360	125	> 250	64
UNI13121	302	32	NT	> 250
UNI13122	274	250	NT	> 250
UNI13123	296	250	NT	>250
UNI13138	357	8	NT	8
UNI13139	322	8	NT	16
UNI13140	255	250	NT	32
UNI13142	290	8	NT	4
UNI13145	416	32	NT	32
UNI13146	300	16	8	8
UNI13147	316	16	4	2
UNI13150	332	0.125	NT	NT
UNI13151	350	0.125	NT	NT
UNI13153	303	2	NT	4
UNI13157	350	> 250	NT	125
UNI13168	347	125	NT	64

Table S4. Compounds confirmed as hits in the Gram-negative panel. MIC values ≤ 8 µg/mL (green); between 16 and 32 µg/mL (yellow); ≥ 64 µg/mL (red); > 250: MIC was not determined in the concentration range tested; NT: compound was not tested in the bacterial isolate.

		Bacterial Isolates (MIC µg/mL)												
Compound ID	**Molecular weight (g/mol)**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
ATx201 (Niclosamide)	327	> 250	> 250	> 250	> 250	> 250	> 250	> 250	> 250	> 250	> 250	> 250	> 250	0.25
ATx11013	389	>250	>250	>250	32	>250	>250	>250	>250	>250	>250	>250	>250	NT
ATx11015	357	>250	>250	> 250	125	>250	> 250	>250	>250	>250	>250	>250	>250	NT
ATx11021	424	>250	>250	>250	32	>250	>250	>250	>250	>250	>250	>250	>250	NT
ATx11034	220	250	250	250	250	250	> 250	NT	NT	250	250	250	250	NT
ATx11035	245	250	250	250	250	250	> 250	NT	NT	> 250	250	> 250	> 250	NT
ATx11039	208	> 250	250	NT	250	> 250	> 250	NT	NT	> 250	250	> 250	> 250	NT
ATx11054	248	250	> 250	> 250	> 250	> 250	> 250	NT	NT	> 250	> 250	> 250	> 250	NT
ATx11056	278	250	250	> 250	> 250	> 250	> 250	NT	NT	> 250	250	> 250	> 250	NT
ATx11057	273	250	250	> 250	250	250	> 250	NT	NT	> 250	250	> 250	> 250	NT
ATx11058	259	250	250	> 250	250	250	> 250	NT	NT	> 250	> 250	> 250	250	NT
ATx11067	310	250	> 250	> 250	250	250	> 250	NT	NT	> 250	> 250	> 250	> 250	NT
ATx11070	277	> 250	> 250	> 250	250	250	> 250	NT	NT	NT	NT	NT	NT	NT
ATx11071	169	250	> 250	> 250	> 250	> 250	> 250	NT	NT	> 250	250	> 250	> 250	NT

ATx11168	384	> 250	> 250	250	> 250	> 250	> 250	NT	NT	> 250	> 250	> 250	> 250	NT
ATx11173	292	250	250	250	250	250	> 250	NT	NT	> 250	> 250	> 250	> 250	NT
ATx11174	294	250	> 250	250	250	250	> 250	NT	NT	> 250	> 250	> 250	> 250	NT
ATx11182	260	> 250	> 250	> 250	250	> 250	> 250	NT	NT	> 250	> 250	> 250	> 250	NT
ATx11184	257	> 250	NT	NT	> 250	> 250	250	125	250	NT	NT	NT	NT	NT
ATx11197	200	250	250	250	250	250	> 250	NT	NT	250	250	250	250	NT
ATx11217	260	250	250	250	250	250	> 250	NT	NT	250	250	250	250	NT
ATx11272	241	250	> 250	> 250	250	125	> 250	NT	NT	> 250	> 250	> 250	> 250	NT
ATx11282	251	> 250	> 250	125	> 250	> 250	> 250	NT	NT	> 250	> 250	> 250	NT	NT
ATx11284	249	250	250	125	250	125	> 250	NT	NT	> 250	> 250	> 250	> 250	NT
ATx11291	251	250	> 250	125	250	250	> 250	NT	NT	> 250	> 250	> 250	> 250	NT
ATx11332	255	250	250	125	250	125	> 250	NT	NT	NT	NT	NT	NT	NT
ATx11334	240	250	> 250	125	250	> 250	> 250	NT	NT	> 250	> 250	> 250	> 250	NT
ATx11432	466	250	250	125	250	125	> 250	NT	NT	NT	NT	NT	NT	NT
ATx11434	293	250	> 250	125	250	> 250	> 250	NT	NT	> 250	> 250	> 250	> 250	NT
ATx11436	237	250	> 250	125	250	> 250	> 250	NT	NT	> 250	> 250	> 250	> 250	NT
ATx12000	663	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	32
ATx12001	441	250	250	250	250	250	>250	>250	>250	>250	>250	>250	>250	2

ATx12004	449	64	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	NT
ATx12005	626	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	4
UNI12007	314	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	<0.125
UNI12008	248	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	4
UNI12010	847	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	<0.125
UNI12011	388	> 125	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	4
ATx13023	340	250	> 250	> 250	> 250	> 250	> 250	NT	NT	> 250	> 250	> 250	> 250	NT
ATx13049	279	250	> 250	> 250	250	> 250	> 250	NT	NT	> 250	> 250	> 250	> 250	NT
UNI13050	315	> 250	> 250	> 250	> 250	> 250	> 250	> 250	> 250	> 250	> 250	> 250	> 250	0.125
ATx13055	317	> 250	> 250	> 250	250	> 250	> 250	NT	NT	NT	NT	> 250	> 250	NT
ATx13056	361	250	> 250	> 250	250	> 250	> 250	NT	NT	NT	NT	> 250	> 250	16
UNI13098	289	> 250	NT	NT	> 250	> 250	> 250	NT	NT	NT	NT	NT	NT	250
UNI13101	327	> 250	NT	NT	> 250	> 250	> 250	NT	NT	NT	NT	NT	NT	32
UNI13103	326	> 250	NT	NT	> 250	> 250	> 250	NT	NT	NT	NT	NT	NT	16
UNI13106	296	> 250	NT	NT	> 250	> 250	> 250	NT	NT	NT	NT	NT	NT	250
UNI13113	375	> 250	NT	NT	> 250	> 250	250	NT	NT	NT	NT	NT	NT	NT
UNI13121	302	> 250	> 250	> 250	NT	NT	NT	NT	NT	NT	NT	NT	NT	4
UNI13138	357	> 250	> 250	> 250	NT	NT	NT	NT	NT	NT	NT	NT	NT	4

UNI13139	322	> 250	> 250	> 250	NT	NT	NT	NT	NT	NT	NT	NT	NT	2
UNI13140	255	> 250	> 250	> 250	NT	NT	NT	NT	NT	NT	NT	NT	NT	250
UNI13142	291	> 250	> 250	> 250	NT	NT	NT	NT	NT	NT	NT	NT	NT	8
UNI13145	416	> 250	> 250	> 250	NT	NT	NT	NT	NT	NT	NT	NT	NT	2
UNI13146	300	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	4
UNI13147	317	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	4
UNI13149	301	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	16
UNI13153	304	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	8
UNI13157	350	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	16
UNI13160	325	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	32
UNI13161	313	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	8
UNI13163	303	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	16
UNI13168	347	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	8
UNI13172	328	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	8

1. *E. coli* WT 6252; **2.** *E. coli* R10709; **3.** *E. coli* KBM16; **4.** *E. coli* JW045-3 (ΔacrA); **5.** *E. coli* JW0451-2 (ΔacrB); **6.** *P. aeruginosa* WT 76610; **7.** *P. aeruginosa* 9328; **8.** *P. aeruginosa* GW; **9.** *K. pneumoniae* WT 7725; **10.** *K. pneumoniae* MONT; **11.** *K. pneumoniae* 2303; **12.** *A. baumannii* T23; **13.** *H. pylori* ATCC 43504

Table S5. ATx201 and ATx13050 *in vitro* susceptibility of *staphylococci* (n = 11), *enterococci* (n = 54) and *H. pylori* clinical isolates (n = 49).

	MIC (µg/mL)	
Strains ID	**ATx13050**	**ATx201**
S. aureus Newman	0.5	0.25
MRSA 02	> 125	0.25
MRSA 03	> 125	0.5
MRSA 10	2	0.25
MRSA 49	1	0.125
MRSA 98	> 125	0.5
MRSA 103	> 125	0.25
S. aureus K00086	> 125	0.5
S. aureus K000796	4	0.25
S. aureus mupA 13	1	0.5
S. aureus ATCC 29213	2	0.5
	MIC (µg/mL)	
Strains ID	**ATx13050**	**ATx201**
VRE V5	0.125	> 125
VRE V34	0.125	> 125
VRE V42	0.125	0.125
VRE V55	0.125	0.125
VRE V59	0.125	1
VRE V77	0.125	0.125
VRE V95	0.125	0.125
VRE V140	0.125	0.125
VRE V171	> 125	0.125
VRE V181	0.25	0.25
E. faecium E0084	1	1

E. faecium E0088	2	4
E. faecium E0097	16	8
E. faecium E0100	0.125	0.125
E. faecium E0157	16	8
E. faecium E0745	0.125	0.125
E. faecium E0804	0.25	0.125
E. faecium E0810	4	8
E. faecium E1071	0.5	0.5
E. faecium E1162	2	0.5
E. faecium E1583	0.25	0.5
E. faecium E1716	0.25	0.25
E. faecium E1717	8	8
E. faecium E1721	8	8
E. faecium E1764	8	8
E. faecium E1780	8	8
E. faecium E4244	8	4
E. faecium E4344	0.5	0.5
E. faecium E4425	4	2
E. faecium E4426	8	2
E. faecium E6009	1	4
E. faecium E6035	1	2
E. faecium E6036	8	0.25
E. faecium E7170	8	4
E. faecium E7192	2	4
E. faecium E7333	2	8
E. faecium E7424	4	4
E. faecium E7641	2	4

E. faecium E7842	2	8
E. faecium E7874	4	0.5
E. faecium E7876	0.5	4
E. faecium E8028	2	4
E. faecium E8042	0.5	1
E. faecium E8043	1	2
E. faecium E8202	2	1
E. faecium E8436	2	4
E. faecium E8437	4	4
E. faecium E8444	0.125	0.125
E. faecium E8460	4	2
E. faecium E8671	8	0.25
E. faecium E9004	4	4
E. faecium E9006	4	4
E. faecium E9029	4	2
E. faecalis ATCC 29212	0.5	0.25

	MIC (µg/mL)	
Strains ID	**ATx13050**	**ATx201**
H. pylori 008A	< 0.125	0.125
H. pylori 013A	< 0.125	< 0.125
H. pylori 020A	0.125	0.5
H. pylori 024K	< 0.125	0.25
H. pylori 025A	0.125	1
H. pylori 028K	< 0.125	0.5
H. pylori 030A	< 0.125	1
H. pylori 046A	0.125	0.25
H. pylori 055A	< 0.125	< 0.125

H. pylori 071k	< 0.125	0.125
H. pylori 073k	< 0.125	< 0.125
H. pylori 084K	0.125	0.5
H. pylori 091k	0.125	0.5
H. pylori 102k	< 0.125	< 0.125
H. pylori 108A	0.125	0.25
H. pylori 114k	< 0.125	0.5
H. pylori 130k	0.125	0.125
H. pylori 1353	0.125	0.5
H. pylori 1427	< 0.125	0.5
H. pylori 1436	0.125	1
H. pylori 1494	< 0.125	0.25
H. pylori 2237	0.125	0.125
H. pylori 2238	< 0.125	1
H. pylori 2278	< 0.125	0.25
H. pylori 2390	0.125	0.5
H. pylori 2507	0.125	0.25
H. pylori 2581	0.125	0.25
H. pylori 2589	0.125	1
H. pylori 25A	0.125	0.125
H. pylori 2797	0.125	0.125
H. pylori 27A	< 0.125	0.125
H. pylori 3127	0.25	0.125
H. pylori 3175	0.125	0.25
H. pylori 3207	0.125	0.125
H. pylori 3250	0.5	0.125
H. pylori 3748	< 0.125	0.125

H. pylori 379935-3	0.125	0.25
H. pylori 467	0.5	0.5
H. pylori 573	< 0.125	0.25
H. pylori 585	0.125	0.125
H. pylori 74A	0.125	0.5
H. pylori 76k	0.125	< 0.125
H. pylori 906	0.25	0.125
H. pylori 95k	0.25	0.25
H. pylori 99A	0.25	0.125
H. pylori AK043	0.5	0.5
H. pylori GU009K	1	0.25
H. pylori GU010A	0.25	0.25
H. pylori ATCC 43504	0.125	0.25

CHAPTER IV – MANUSCRIPT III

GENOMIC EPIDEMIOLOGY AND CHARACTERIZATION OF ANTIMICROBIAL RESISTANCE PROFILE OF *STAPHYLOCOCCUS AUREUS* ISOLATES ASSOCIATED WITH BOVINE SUBCLINICAL MASTITIS

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Abstract

Veterinary settings can act as a reservoir for resistant pathogens contributing to the antimicrobial resistance crisis. *Staphylococcus aureus* is an important pathogen that causes diseases both in humans and animals. As well, *S. aureus* constitutes a major public health concern, due to its ability to develop resistance towards a variety of antibiotics. In dairy cows, *S. aureus* is one of the most important pathogens involved in contagious mastitis.

This study aimed to determine the phenotypic and genotypic susceptibility profile of 59 *S. aureus* isolates collected from the teat skin and milk of dairy cows diagnosed with subclinical mastitis. Minimal inhibitory concentrations (MICs) of multiple antibiotics used in the clinical and veterinary practices, were determined for all isolates. We used WGS (whole genome sequencing) tools to identify the presence of specific genes described to confer resistance to the antibiotics included in the study and the isolates further characterized in regard to multi-locus sequence typing (MLST), spa-typing and presence of virulence genes.

All isolates were identified as multidrug resistant by phenotypic screening, displaying resistance towards antibiotics used both in the clinic and veterinary practice and the findings correlated with identified resistance genes. Different sequence types were identified, being ST151 the most prevalent among both groups, but in general, isolates from teat skin and milk, were of a similar genetic background. As well, all isolates carried virulence genes related with toxin production, that can ultimately cause host tissue damage.

Our isolates showed a high level of antimicrobial resistance. Although the MLST lineages observed are considered cattle specific, these multi-resistant isolates represent a reservoir of antibiotic resistance genes that could contribute to the dissemination of antibiotic resistance.

Keywords: *Staphylococcus aureus,* antimicrobial susceptibility, antimicrobial resistance MLST, One Health, bovine mastitis, whole-genome sequence

Introduction

Antimicrobial resistance is a global public health concern that complicates the treatment of several infectious diseases [1]. Multiple factors can contribute considerably to this phenomenon as well for the increasing incidence of multidrug resistant pathogens, including the use of antimicrobial agents in veterinary settings, where antibiotics are used in a larger quantities compared to human clinical use [2,3]. The continuous use of antibiotics in veterinary settings, either for therapy, prevention or growth promotion, can contribute to the selection of antibiotic resistance, that ultimately leads to the transmission of resistant pathogens or resistance genes from the environment into the clinic [3–5].

Staphylococcus aureus is an important and versatile human and veterinary pathogen causing a wide range of systemic infectious diseases and a variety of acute and chronic infections [6]. In dairy cows, it is considered one of the most important pathogens related to contagious mastitis [7], an infectious disease that causes the inflammation of the mammary gland and constitutes a significant economic burden due to production lost [8,9]. *S. aureus* is also known for having the ability to acquire resistance to different antimicrobial classes, including, ß-lactams, macrolides, fluoroquinolones, tetracyclines and more recently to glycopeptides [10]. Furthermore, different studies reported the presence of virulence genes in *S. aureus* isolates from subclinical mastitis and milk [9,12,13] many of them encoding for toxins that can be harmful to humans, such as toxic shock syndrome toxin-1 and leukocidins that are heat stable and often associated with food poisoning [14,15]. Altogether these factors are contributing to *S. aureus* ability to cause disease in humans.

To date different sequence types (STs) and spa-types of *S. aureus*, have been described, allowing to discriminate human from animal associated clones. In fact, several STs have been linked with livestock, specifically with cattle and bovine mastitis across the world [9,16]. Although the overall impact of animal-human transmission is reported to be low [17], the occurrence of multidrug resistant bacteria in animals can still pose a risk for public health [18–21].

Different studies focusing on the susceptibility patterns of *S. aureus* isolates collected from livestock and dairy cattle environments, were carried out in the past and susceptibility shown to vary depending on geographical location [22,23]. For instance, in southern Europe higher rates of antibiotic resistance are reported, specially towards ß-lactams, erythromycin and tetracyclines [24–26]. Whereas in Northern Europe, in countries such as Denmark, Sweden and Norway resistance to these antibiotics tend to be lower [8,23,27], in Germany high rates of resistance towards tetracyclines, erythromycin, clindamycin and ß-lactams were reported [28].

To the best of our knowledge, the susceptibility patterns and genomic characterization of antibiotic resistance genes of *S. aureus* isolates from dairy cows with subclinical mastitis has not been investigated particularly, in dairy herds with automatic milking systems (AMS).

In AMS milking hygiene and teat skin cleaning may be distinctive from conventional milking systems [29]. Furthermore, it allows to milk the cows several times a day without contact with human hands, and several cows can be milked by the same milking unit. Combined, these factors may affect both the teat skin colonization, the transmission dynamic or pathways and, epidemiology of contagious mastitis pathogens [30].

The objective of the present study was to characterize the phenotypic and genotypic resistance patterns of *S. aureus* isolates from milk and the teat skin samples of dairy cows and, to relate this with resistance and virulence profiles.

Materials and Methods

Bacterial isolates

In total 59 bovine *S. aureus* isolates were selected for the present study, representing both teat skin (n = 30) and milk (n = 29) origins. The bacterial isolates were harvested from milk and teat skin samples collected from lactating cows in 8 AMS dairy herds participating in a national research project called “STOPMAST” for investigation of the epidemiology of contagious mastitis pathogens in Danish dairy herds [7]. Selection of dairy herds for that project was based on

the criteria of having at least three automatic milking units, a bulk tank positive *Streptococcus agalactiae* status, and a willingness from the farmer to participate. The cow-level selection criteria were somatic cell count higher than 200,000 cells/mL at the previous monthly routine sampling (subclinical mastitis), and no antibiotic treatment in the 4 weeks prior to sample collection. The samples were collected between February and May 2017. Teat skin swabs were collected using the modified wet-dry method and milk samples were taken aseptically for bacterial culture [7,31].

S. aureus were identified using conventional culture and biochemical methods and were then confirmed by the MALDI-TOF assay [31]. Isolates included in this study were randomly selected among available isolates.

Antimicrobial susceptibility testing

The minimum inhibitory concentration (MIC) of each antibiotic was determined by the microbroth dilution method in accordance to the guidelines established by the Clinical and Laboratory Standards Institute (CLSI) [32]. All 59 *S. aureus* isolates were screened in duplicate and at two different time points for the following antibiotics; penicillin, oxacillin, cloxacillin, amoxicillin, erythromycin, clindamycin, norfloxacin, enrofloxacin, tetracycline, fusidic acid, linezolid, and trimethoprim and sulfamethoxazole combinations. Antibiotics were selected based on their importance for veterinary and clinical practice as well as representing different available antibiotic classes. *S. aureus* strain ATCC 29213, was included for quality control purposes.

Bacterial isolates were characterized as susceptible or resistant based on the breakpoints available in the CLSI guidelines or, for the ones not included, as it is the case of cloxacillin, amoxicillin, enrofloxacin and fusidic acid, breakpoints were assessed based on EUCAST (European Committee for Antimicrobial Susceptibility Testing) breakpoints [33] or other relevant sources [34,35].

Whole-genome sequencing and analysis

Whole-genome sequencing of the 59 isolates was conducted by BaseClear BV. Briefly, 59 isolates were sent to BaseClear, who extracted genomic DNA using standard molecular biology kits from

ZYMO and prepared the genomic libraries with Illumina genomic Nextera XT library. Paired end reads, quality control and quantification were generated using Illumina HISeq2500. FASTQ read sequence files were generated using bcl2fastq2 version 2.18. Sequencing stats and quality filtering can be found in supplementary data (Table S1). Quality-filtered sequences were assembled using SPAdes v3.13.0 36 with default parameters.

Identification of resistance and virulence related genes

The presence of antibiotic resistance genes (ARGs) was investigated using protein sequences obtained from UNiProtKB (Table 1). Selection of ARGs was made based on genes being described in the literature to be involved in conferring resistance to the antibiotics selected for the phenotypic screening [13,37–41]. Presence of these genes in our isolates was identified using Prokka 1.13.3 [42].

Furthermore, isolates were investigated for the presence of virulence genes using Virulence Finder 2.0 [43].

Molecular typing and phylogenetic tree construction

Multilocus sequence type (MLST) and *spa* type were investigated by submitting the assembled genomes to MLST v2.0 [44] and spaTyper 1.0 [45], respectively.

Phylogeny *S. aureus* isolates from milk and teat skin was assessed. In addition, *S. aureus* genomes from human and animal origins available on PATRIC database, were selected and included as reference genomes. The phylogenetic tree was generated using the codon tree method within PATRIC 3.6.1. with default parameters [46], which makes use of PGFams (PATRIC's global Protein Families) as homology groups and analyzes aligned proteins and coding DNA from single copy genes using RAxML [47,48]. Resulting tree was visualized with PATRIC visualization tool. Information regarding tree statistics and genomes used as reference can be found in supplementary data (Tables S5, S6 and S7).

Table 1. Antibiotic resistance genes (ARGs) selected and UNiProtKB accession numbers.

Antimicrobial agent	ARGs	UNIProt accession number
ß-lactams		
Penicillin	*blaZ*	R4VFZ6_STAAU
Amoxicillin	*mecA*	O54286_STAAU
Oxacillin	*mecC*	F4NA57_STAAU
Cloxacillin		
Tetracycline	*tet*	TCR_STAAU
	tetL	A0A1B1P6P9_STAAU
	tetM	TETM_STAAU
	tetK	D2JG24_STAAU
	tetO	R4VFZ6_STAAU
Macrolides and Lincosamides	*ermA*	ERMA_STAAU
Erythromycin	*ermB*	Q799N2_STAAU
Clindamycin	*ermC*	ERMC2_STAAU
	msrA	A0A068LCQ4_STAAU
Oxazolidinones	*cfr*	CFR_STAAU
Linezolid		
Fluoroquinolones	*norA*	NORA_STAAU
Norfloxacin	*norB*	NORB_STAAE
Enrofloxacin	*mepA*	MEPA_STAA8
Metabolic Pathway Inhibitors	*folA*	DYR_STAAU
Trimethoprim/Sulfamethoxazole	*folP*	DHPS_STAAM
Fusidic Acid	*fusA*	3EFG_STAAU
	fusB	D3JCX0_STAAU

Statistical analysis

Statistical analysis was performed with GraphPad Prism 8 (GraphPad Software Inc.). Differences in the presence of STs, *spa* types, virulence and resistance genes between isolates from milk and teat skin, were investigated applying the Wilcoxon test and considered significant when $P < 0.05$.

Results

Susceptibility testing

Resistance was equally distributed among isolates from milk and teat skin (Table 2). In both groups, most isolates were resistant to penicillin (65% and 86%), amoxicillin (55% and 76%), oxacillin (96% and 86%) and cloxacillin (65% and 73%), respectively. Furthermore, high resistance rates were found for tetracycline (41% and 56%), erythromycin (65% and 93%), clindamycin (100%), linezolid (100% and 96%), trimethoprim/sulfamethoxazole combinations (96%) and fusidic acid (100%). Whereas lower rates of resistance were found for norfloxacin (7% and 6%), enrofloxacin (31% and 13%) and tetracycline (41% and 56%), respectively. MICs of all tested strains are displayed in Table S2. Based on the phenotypic analysis the isolates were considered to be multidrug resistant (displayed resistance to three or more antibiotics).

Table 2. Distribution of phenotypic antimicrobial resistance of *S. aureus* isolated from cow's milk and teat skin (n = 59). Rates are presented by sampling group.

Antimicrobials	Milk samples (n= 29)	Teat skin samples (n=30)
Penicillin	65%	86%
Amoxicillin	55%	76%
Oxacillin	96%	86%
Cloxacillin	65%	73%
Tetracycline	41%	56%
Erythromycin	65%	93%
Clindamycin	100%	100%
Linezolid	100%	96%
Norfloxacin	7%	6%
Enrofloxacin	31%	13%
Fusidic Acid	100%	100%
Trimethoprim/Sulfamethoxazole	96%	96%

Proportion of resistance genes and relation with phenotypic screening

The presence of specific genes described to confer resistance to the antibiotics included in the phenotypic screening, as described in Table 1, was investigated.

Three isolates out of fifty-nine, were characterized as MRSA by carrying the *mecA* (n = 2) and the *mecC* (n = 1) genes. Furthermore, *norA*, *norB* and *mepA*, involved in fluoroquinolones resistance, were found in all isolates except one. Presence of *ermA* and *ermC*, related with macrolides resistance, varied according to isolates origin. *ermA* was found in 58% and 90% isolates from milk and teat skin, respectively. Whereas *ermC* was only found in 14% of isolates recovered from milk. Additionally, gene *tet* was found in a single isolate retrieved from the teat skin, while *fusB* was found in two isolates from milk. Genes *tetM*, *tetL*, *msrA*, *cfr*, *folA* and *folP* were present in all isolates, regardless origin. Gene *fusA* was found absent together with genes *blaZ*, *ermB*, *tetK* and *tetO* that were not retrieved with our pipeline.

We noted a high discrepancy between the phenotypic screening and genomic analysis of fusidic acid, where a high percentage of the isolates were found to be phenotypically resistant, but genes *fusA* was not retrieved with the implemented targeted pipeline and *fusB* was found only to be present in two isolates, as mentioned before. As well, the phenotypic screening deemed the isolates to be resistant towards the ß-lactam antibiotics included, which was not translated in the genomic analysis.

One other case was the presence of *norA* and *norB*, both quinolones related resistant proteins, whereas low levels of phenotypic resistance were observed (Table 3).

ARGs presence/absence in all isolates are displayed in Table S3.

Table 3. Correlation between the phenotypic screening and genotype prediction.

	Number of isolates resistant by phenotype		Number of isolates susceptible by phenotype	
	Susceptible by genotype	Resistant by genotype	Susceptible by genotype	Resistant by genotype
Penicillin				
mecA	43	2	14	0
mecC	45	0	13	1
Amoxicillin				
mecA	38	2	19	0
mecC	39	0	19	0
Oxacillin				
mecA	46	2	5	0
mecC	54	1	4	0
Cloxacillin				
mecA	42	2	15	0
mecC	44	0	14	1
Tetracycline				
tet	28	1	30	0
tetL	0	29	0	30
tetM	0	29	0	30
Erythromycin				
ermA	15	40	0	4
ermC	52	3	3	1
msrA	0	55	0	4
Clindamycin				
ermA	12	44	0	3
ermC	55	4	0	0
msrA	0	59	0	0
Linezolid				
cfr	0	58	0	1
Norfloxacin				
norA	0	7	1	51
norB	0	7	0	52
mepA	0	7	0	52
Enrofloxacin				
norA	0	16	1	42
norB	0	16	0	43
mepA	0	16	0	43
Trimethoprim/Sulfamethoxazole				
folA	0	57	0	2
folP	0	57	0	2
Fusidic acid				
fusA	59	0	0	0
fusB	56	2	1	0

Virulence genes

A total of 18 virulence genes were identified. Those could be organized in two groups, exoenzymes (*aur*, *splA* and *splB*) and genes related with toxin production (*hlgA/B/C*, *lukD/E*, *sei/m/n/o/u/g/l/c* and *tst*) (Table 4). Genes involved in biofilm production were not identified. Virulence genes per isolate are displayed in Table S4.

Table 4. Virulence genes distribution (%) across *S. aureus* isolates (n = 59). Percentages were calculated based on sampling group.

	Virulence genes	Milk samples (n = 29)	Teat skin samples (n = 30)
Exoenzymes	*spa*	100%	100%
	aur	100%	100%
	splA	72%	87%
	splB	82%	93%
Toxin production	*hglA*	97%	97%
	hglB	100%	100%
	hglC	100%	100%
	lukD	76%	80%
	lukE	69%	63%
	sei	21%	17%
	sem	24%	20%
	sen	24%	20%
	seo	24%	13%
	seu	21%	13%
	seg	14%	17%
	tst	3%	10%
	sel	7%	10%
	sec	-	10%

MLST and *Spa* typing

MLST typing showed isolates to be distributed in seven groups, with ST151 as the predominant one in both milk (38%) and teat skin (57%) isolates. Distribution of following STs showed variability according to origin. ST3891 (34%) was the second most prevalent in isolates from milk, followed by ST133 (14%), ST504 (10%) and ST479 (0.3%). In the case of isolates from teat skin, ST504 (13%) was the second most prevalent, followed by ST1380 (10%), ST3891 (7%), ST3897 (7%), ST133 (3%) and ST479 (3%) (Figure 1A). Statistical analysis showed no significant difference in distribution of MLST and *spa* types between isolates from milk or teat skin, except for ST1380 and ST3897, that were shown to be associated with teat skin.

Furthermore, *spa* types were also determined. Among all isolates five different spa types were observed. Types t529 (55%), t519 (27%) and t1403 (14%) were found in milk and types t529 (70%), t528 (10%), t543 (7%) and t1403 (3%) were found in isolates from the teat skin.

Furthermore, in total 4 isolates were found to be of unknown *spa* type and, t519 was found to be exclusive of milk, while t528 and t543 were found to be exclusive of teat skin isolates (Figure 1B). The relation between ST and resistance profile of the isolates was assessed (Table 5), showing that resistance towards the antibiotics tested is similarly distributed across isolates.

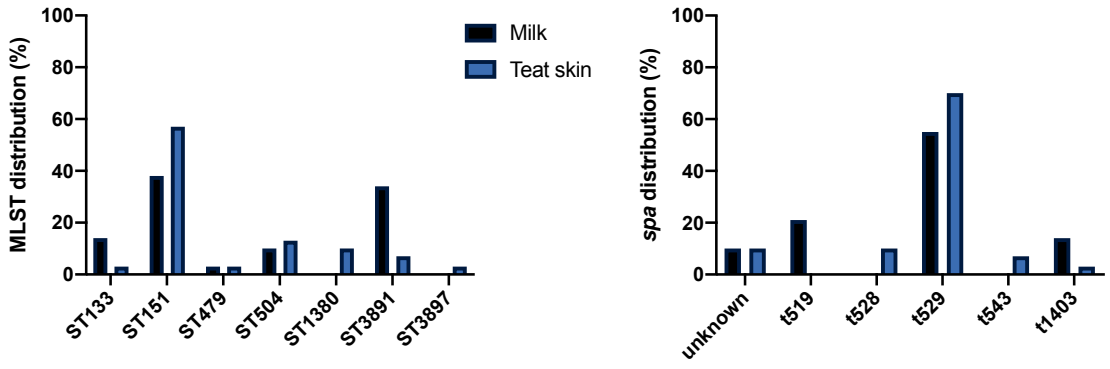

Figure 1. STs (A) and *spa* type (B) distribution (%) among *S. aureus* isolates per sample group.

Table 5. Antibiotic resistance profiles (%) of *S. aureus* isolates in relation to molecular phylogeny based on MLST distribution of the isolates.

Sequence types	Isolates (n)	PEN	AMX	OXA	CLX	TET	ERY	CLD	LZ	NOR	ENR	FA	TS
ST133	5	40%	60%	100%	80%	80%	80%	100%	100%	40%	60%	100%	100%
ST151	28	79%	61%	86%	60%	39%	93%	100%	100%	4%	11%	100%	100%
ST479	2	100%	100%	100%	100%	100%	100%	100%	100%	-	50%	100%	100%
ST504	7	71%	57%	100%	71%	71%	100%	100%	100%	-	14%	100%	100%
ST1380	3	100%	100%	100%	100%	100%	100%	100%	100%	33%	33%	100%	100%
ST3891	11	75%	75%	100%	75%	33%	91%	100%	100%	8%	33%	100%	100%
ST3897	2	100%	100%	100%	100%	50%	100%	100%	100%	-	-	100%	100%

PEN: penicillin; AMX: amoxicillin; OXA: oxacillin; TET: tetracycline; ERY: erythromycin; CLD: clindamycin; LZ: linezolid; NOR: norfloxacin; ENR: enrofloxacin; FA: Fusidic acid; TS: trimethoprim-sulfamethoxazole combinations.

Phylogenetic analysis

The presented phylogenetic tree comprises 78 *S. aureus* genomes. Besides our samples, the tree includes genomes from both animal and human origin, selected as reference genomes. In general, the tree shows that *S. aureus* isolates from teat skin and milk clustered together into groups of identical or closely related STs. The largest cluster in the tree consisted primarily of isolates belonging to ST151 and double locus variant (DLV) ST504. Whereas the remaining clusters consisted of ST3891 isolates (a single locus variant, SLV, of ST50), ST133, ST479 and ST3897 (Figure 2).

Surprisingly, isolates 16, 22 and 683, belonging to ST3891 and identified as MRSA, by both phenotypic and genomic analysis, did not group together with *S. aureus* LGA251 a well characterized MRSA from bovine origin. In contrast isolates belonging to ST133, seemed to be closer related with previously described MRSA isolates from animal and human origins.

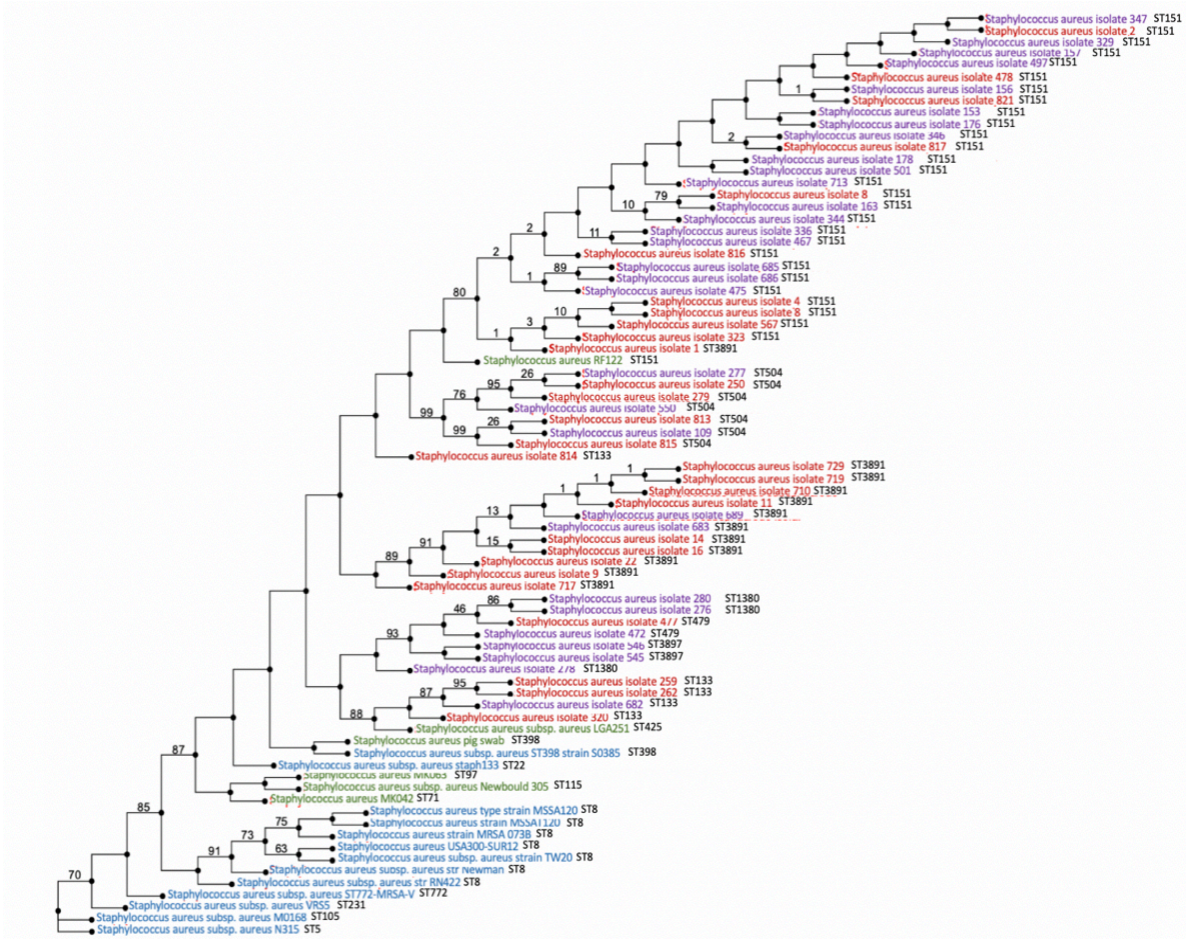

Figure 2. Phylogram of *S. aureus* isolates recovered from milk (red) and teat skin (purple). *S. aureus* isolates from human origin (blue) and animal origin (green) were used as reference genomes. STs are displayed in black. The phylogenetic tree is based on the alignment of 97 PGFams. It shows that *S. aureus* isolates from milk and teat skin group together in closely related STs.

Discussion

From an evolutionary perspective, *S. aureus* can become resistance to different antibiotics through gene mutations, often associated with the antibiotic mode of action, or through the acquisition of genetic mobile elements carrying resistance determinants [49]. However, in some cases antimicrobial resistance can occur without being associated to a genetic event and rather related with persistence, biofilm formation or associated with metabolic state and growth conditions [50].

The examined bacterial isolates revealed a high prevalence of phenotypic resistance towards the antibiotics tested, including antibiotics used in the clinic as a last resort for the treatment of infections caused by *S. aureus*, such as linezolid. And the isolates considered to be multidrug resistant. Although other studies reported *S. aureus* high resistance rates [13,51], in Europe reports of linezolid resistance in *S. aureus* remains low [43,52]. In our study, linezolid resistance was found in 96% of isolates collected from milk and 100% of the isolates from teat skin and associated with the presence of the *cfr* gene that encodes for a ribosomal RNA large subunit methyltransferase and seems to be related with resistance to different antibiotics by altering the antibiotic target [53].

Our bacterial isolates showed also resistance towards trimethoprim/sulfamethoxazole combinations, tetracycline, erythromycin and clindamycin. This was also, confirmed by the presence of genes *folA* and *folP, tet, tetM, tetL, ermA, ermC, msrA*, respectively. Opposite to tetracycline and erythromycin, clindamycin and trimethoprim/sulfamethoxazole combinations are still used in Denmark for the treatment mastitis in dairy cows [54], which may explain the resistance observed.

In the case of fusidic acid, the antimicrobial resistance was observed phenotypically, but *fusB* was only observed in one isolate from milk and two isolates from the teat skin, and *fusA* was not detected with the implemented pipeline.

In addition, low rates of phenotypic resistance were observed for norfloxacin and enrofloxacin. However, genes associated with fluoroquinolones resistance, were present in all isolates except one *norA* and *norB* genes are members of multidrug efflux pumps, that are described to contribute

to resistance towards multiple antibiotics in *S. aureus,* including fluoroquinolones, when up-regulated [55,56]. Although in Denmark, fluoroquinolones can only be prescribed in veterinary settings as a last-line antibiotic, still the use of enrofloxacin, to treat environmental mastitis caused by *Escherichia co*li could potentially explain the cases of resistance observed [54]. As well, other studies suggest that resistance towards fluoroquinolones could be related with the widespread use of biocides for hygiene purposes, such as cleaning the milking machines, which may promote cross-resistance, since the mechanism conferring resistance is mediated by the norA efflux pump in both cases [9,57]. Finally, a high percentage of the bacterial isolates was also resistant to all penicillins and ß-lactams screened, which came to no surprise, since according to DANMAP, penicillins are widely used in dairy farms for the treatment of bovine mastitis in dairy herds [54].

Based on the phenotypic screening, 55 isolates were classified as MRSA, due to displayed phenotypic resistance towards oxacillin. However, only three isolates carried the *mecA* and *mecC* genes, considered the golden standard for MRSA detection [58]. Similarly, the *blaZ* gene that confers resistance to penicillins, was not retrieved with the implemented pipeline. Furthermore, Liu et al [13] reported *S. aureus* isolates from humans and animals to be phenotypically resistant to penicillin and other ß-lactams but lacking the *blaZ* and *mecA* genes.

The fact that some genes were not retrieved with our pipeline could also be a limitation to our pipeline, which makes use of protein sequences to identify the gene of interest within the genome, meaning that the gene may be considered to be absent (cases of *mecA* and *fusA*) or not retrieved as it was the case of genes *blaZ, ermB, tetK and tetO* in case of mutations. In a similar study, Gordon et al [40] also reported a high discrepancy between ß-lactams (e.g. penicillin) and fusidic acid phenotypic resistance profile and the presence of their respective resistance genes.

Nevertheless, the fact that antibiotic resistance is a multifactorial event and resistance towards one antibiotic or antibiotic class can occur through different mechanisms or associated with different resistance genes [59,60] can also be a possible explanation for the discrepancies observed between phenotypic and genomic resistance, since a limited amount of genes based on the primary

mechanisms of resistance of *S. aureus* towards the selected antibiotics were investigated. On the other hand, phenotypic resistance related with bacteria transiently becoming resistant to antibiotics without a genetic event, has been reported before and associated with bacteria metabolic state and found to be particularly relevant in the case of persistent infectious diseases, such as mastitis [50,59,61].

While lineage and host-associated genes were identified in the past, human and animal related *S. aureus* were shown to be genetically similar, having a great potential to adapt to different environments. In fact, several STs have been reported to be associated both with bovine mastitis and human colonization and/or infection, including ST1, ST8, ST97 and ST398, although these STs were not identified within our samples, there is the concern that bovine-adapted lineages, such ST133 and ST151, would adapt and develop the ability to spread in both host populations [21,62]. Moreover, isolates belonging to ST133 lineage were already recovered from individuals working in close contact with livestock [25].

In our sample, ST151 was the most prevalent MLST type, which is documented to be associated with bovine mastitis across Europe [63]. To our knowledge, no MRSA strains were associated with this clonal type before, suggesting that, not only clonal variability can occur among MRSA isolates, but also that gene transmission between isolates could be happening. Additionally, all isolates carried several genes encoding for different virulence factors including tst (toxic shock syndrome), leukocidins *lukD* and *LukE*, different enterotoxins (SE) and hemolysins (*hglA-C*), which ultimately can promote bacterial spread and cause damage of the host tissues.

Although our isolates belong to STs, which are considered to be cattle or animal specific, it could potentially act as reservoirs for antimicrobials resistance genes and contribute to its dissemination to other animal species, food chain, and environment since they still share similarities with bacterial isolates of human origin having the potential to exchange pathogenic determinants [21,64,65].

Conclusions

In summary, our results suggest that dairy cows can act as reservoirs of multidrug resistant pathogens, such as *S. aureus*. The proportion of phenotypic antibiotic resistance was in general high and, although discrepancies were observed between resistance phenotype and genotype, in most cases, resistance phenotype was accompanied by the respective genotype. Nevertheless, further investigation is necessary, since understanding the mechanisms underlying resistance phenotype and genotype and respective discrepancies, can contribute to establish novel antibiotic therapies or improve existing ones.

Conflicts of interest statement

All authors declare no conflicts of interest.

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Supplementary Material

Table S1. Sequencing quality statistics after demultiplexing and filtering.

Isolate ID	Number of reads	Yield	Average quality
1	2456267	615	38.45
2	2288189	572	38.50
4	2555669	639	38.41
8	2358212	590	38.66
9	1323153	331	38.38
11	1943871	487	38.44
14	1806474	452	38.45
16	2126219	532	38.52
22	2486999	621	38.62
109	1893575	474	38.38
153	1429000	358	38.29
156	1169520	293	37.97
157	1752050	439	38.37
163	1239110	310	38.47
176	2234398	559	38.36
178	1364759	341	38.74
250	2771472	694	38.66
259	2425944	607	38.58
262	2228855	557	38.63
276	1075889	269	38.54
277	2343710	587	38.66
278	1485597	373	37.95
279	1543819	387	38.30
280	1695759	425	38.33
320	2219329	555	38.42
323	2107584	527	38.42
329	2636807	656	38.34
336	2209564	553	38.46
344	1396257	349	38.51
346	3116152	779	38.44
347	2935732	732	38.61
467	1907316	475	38.50
472	2596455	649	38.56
475	2136859	535	38.27
477	1646307	412	38.23

478	1814644	455	38.23
497	2077972	520	38.25
501	2231187	557	38.27
545	2627555	654	38.28
546	3130421	779	38.59
550	2260022	563	38.58
567	2638576	659	38.47
682	2578545	644	38.48
683	2707900	672	38.58
685	3545069	883	38.66
686	1615543	404	38.27
689	1873376	469	38.26
710	1416722	355	38.07
713	1342323	336	38.21
717	2201498	549	38.41
719	1481084	368	38.20
729	2171129	542	38.45
813	2124321	531	38.48
814	1404609	351	38.24
815	1196926	299	38.28
816	1973168	493	38.41
817	1406940	352	38.14
818	2215233	555	38.40
821	1068992	268	38.18

Table S2. Characterization of *S. aureus* isolates from milk and teat skin and *in vitro* susceptibility testing (MIC in µg/ml). ATCC 29213 was used as quality control.
> 16: mic was not determined in the concentration range tested.

Isolate ID	Origin	MLST	spa	PEN	AMX	CLX	OXA	ENR	NOR	ERY	CLD	TS	TET	LZ	FA
1	milk	3891	t519	> 16	> 16	> 16	1	0.25	1	8	1	> 16	0.25	> 16	8
2	milk	151	t529	>16	16	> 16	16	0.5	2	4	> 16	> 16	0.5	16	16
4	milk	151	t529	0.25	0.125	0.125	16	0.125	1	4	16	> 16	2	16	> 16
8	milk	151	t529	>16	> 16	> 16	16	16	0.5	> 16	> 16	> 16	> 16	> 16	16
9	milk	3891	t519	>16	> 16	> 16	16	0.125	1	8	> 16	> 16	0.5	> 16	> 16
11	milk	3891	t519	>16	16	> 16	4	0.125	1	8	> 16	> 16	0.5	16	16
14	milk	3891	t519	>16	> 16	> 16	16	0.125	0.5	8	> 16	> 16	0.5	> 16	> 16
16	milk	3891	t519	>16	> 16	> 16	16	2	4	0.25	> 16	> 16	1	> 16	16
22	milk	3891	t519	>16	> 16	> 16	> 16	1	2	8	> 16	> 16	1	> 16	> 16
109	teat	504	t529	>16	> 16	> 16	> 16	0.25	0.5	> 16	> 16	> 16	16	> 16	> 16
153	teat	151	t529	>16	> 16	> 16	0.125	0.125	1	8	16	> 16	1	16	16
156	teat	151	t529	>16	16	> 16	16	2	1	8	> 16	> 16	0.5	> 16	> 16
157	teat	151	t529	0.125	0.125	0.125	16	0.125	1	8	> 16	8	0.25	16	16
163	teat	151	t529	>16	> 16	0.125	0.25	0.125	0.125	0.125	16	> 16	0.125	16	16
176	teat	151	t529	>16	0.125	0.125	0.125	0.125	0.25	16	16	> 16	2	2	0.5
178	teat	151	t529	>16	16	> 16	0.125	0.125	0.125	0.125	16	> 16	0.125	> 16	> 16
250	milk	504	t529	>16	0.125	> 16	> 16	0.25	1	> 16	> 16	16	16	> 16	> 16
259	milk	133	t1403	>16	16	>16	> 16	> 16	> 16	> 16	> 16	> 16	16	> 16	> 16
262	milk	133	t1403	>16	16	>16	> 16	0.125	0.5	8	> 16	>16	> 16	> 16	> 16
276	teat	1380	t528	>16	> 16	> 16	> 16	4	8	> 16	> 16	> 16	> 16	> 16	> 16
277	teat	504	t529	>16	> 16	> 16	> 16	0.125	1	16	> 16	> 16	4	> 16	> 16
278	teat	1380	t528	>16	> 16	> 16	> 16	0.125	0.25	> 16	> 16	> 16	> 16	> 16	> 16

279	teat	504	t529	>16	> 16	> 16	> 16	0.5	0.5	> 16	> 16	> 16	> 16	> 16	> 16
280	teat	1380	t528	>16	>16	>16	> 16	0.25	0.5	16	> 16	>16	16	> 16	> 16
320	milk	133	t1403	0.125	0.125	>16	> 16	> 16	0.5	> 16	> 16	>16	> 16	> 16	> 16
323	milk	151	t529	>16	>16	>16	> 16	0.25	0.5	8	> 16	>16	0.5	> 16	16
329	teat	151	t529	>16	>16	>16	> 16	0.25	0.125	> 16	> 16	>16	> 16	> 16	> 16
336	teat	151	t529	2	0.125	0.125	> 16	0.125	0.5	8	> 16	8	0.25	> 16	> 16
344	teat	151	t529	>16	>16	>16	> 16	0.125	0.125	> 16	> 16	>16	16	> 16	16
346	teat	151	t529	>16	>16	>16	> 16	0.125	0.25	> 16	> 16	>16	0.5	> 16	> 16
347	teat	151	t529	>16	0.125	0.125	> 16	0.125	0.5	16	> 16	>16	0.5	> 16	> 16
467	teat	151	t529	>16	4	>16	> 16	0.125	0.25	> 16	> 16	>16	> 16	> 16	> 16
472	teat	479	unknown	16	>16	>16	> 16	0.125	2	> 16	16	>16	> 16	16	> 16
475	teat	151	t529	>16	>16	>16	> 16	0.125	0.125	8	> 16	>16	> 16	> 16	> 16
477	milk	479	unknown	16	>16	>16	> 16	2	4	> 16	> 16	>16	16	> 16	> 16
478	milk	151	t529	>16	0.125	>16	> 16	0.5	1	8	> 16	16	2	> 16	> 16
497	teat	151	t529	>16	>16	>16	> 16	0.125	1	> 16	> 16	>16	> 16	> 16	> 16
501	teat	151	t529	>16	4	>16	> 16	0.125	0.125	8	> 16	>16	0.5	> 16	> 16
545	teat	3897	t543	16	>16	8	> 16	0.25	1	8	> 16	>16	1	> 16	> 16
546	teat	3897	t543	>16	>16	>16	> 16	1	1	> 16	> 16	>16	16	> 16	> 16
550	teat	504	t529	>16	16	>16	> 16	0.125	1	8	> 16	>16	0.5	> 16	> 16
567	milk	151	t529	>16	16	2	> 16	0.25	0.5	> 16	> 16	>16	> 16	> 16	> 16
682	teat	133	t1403	0.125	0.125	0.125	> 16	> 16	> 16	> 16	> 16	>16	> 16	> 16	> 16
683	teat	3891	unknown	0.125	0.125	0.125	> 16	> 16	0.125	> 16	> 16	>16	> 16	> 16	> 16
685	teat	151	t529	0.125	0.125	0.125	> 16	0.125	> 16	> 16	> 16	>16	> 16	> 16	> 16
686	teat	151	t529	16	>16	16	> 16	0.125	1	8	> 16	>16	16	> 16	> 16
689	teat	3891	unknown	>16	>16	16	> 16	0.25	0.25	> 16	> 16	>16	16	> 16	> 16

710	milk	3891	unknown	0.125	0.125	0.125	> 16	> 16	1	16	> 16	>16	> 16	> 16	> 16
713	milk	151	t529	16	>16	>16	> 16	0.125	2	> 16	> 16	>16	2	> 16	> 16
717	milk	3891	t529	0.125	0.125	0.125	> 16	0.125	1	8	> 16	>16	1	> 16	> 16
719	milk	3891	unknown	>16	8	>16	> 16	> 16	> 16	16	> 16	>16	16	> 16	> 16
729	milk	3891	t529	1	1	16	> 16	0.125	0.5	8	> 16	>16	1	> 16	> 16
813	milk	504	t529	0.125	0.125	0.125	> 16	0.5	1	> 16	> 16	>16	> 16	> 16	> 16
814	milk	133	t1403	0.125	8	>16	> 16	0.125	0.5	0.125	> 16	>16	0.125	> 16	> 16
815	milk	504	t529	0.125	0.125	0.125	> 16	> 16	1	8	> 16	16	2	> 16	> 16
816	milk	151	t529	0.125	0.125	0.125	> 16	0.125	1	8	> 16	>16	> 16	> 16	> 16
817	milk	151	t529	0.125	0.125	0.125	> 16	0.125	1	> 16	> 16	16	16	> 16	> 16
818	milk	151	t529	0.125	0.125	0.125	> 16	> 16	0.5	8	> 16	16	0.5	> 16	> 16
821	milk	151	t529	0.125	0.125	0.25	> 16	1	0.5	4	> 16	16	0.5	> 16	> 16
ATCC 29213	-	-	-	0.125	0.25	0.25	0.125	0.125	0.5	0.25	0.125	0.5	0.125	1	0.125

PEN: penicillin; AMX: amoxicillin; CLX: cloxacillin; OXA: oxacillin; ENR: enrofloxacin; NOR: norfloxacin; ERY: erythromycin; CLD: clindamycin; TS: trimethoprim/sulfamethoxazole; TET: tetracycline; LZ: linezolid; FA: fusidic acid

Table S3. Antibiotic resistance genes identified by the target approach.

Isolate ID	*blaZ*	*mecA*	*mecC*	*norA*	*norB*	*mepA*	*ermA*	*ermB*	*ermC*	*msrA*	*folA*	*folP*	*tet*	*tetK*	*tetL*	*tetM*	*tetO*	*cfr*	*fusA*	*fusB*
1	NR	A	A	P	P	P	A	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
2	NR	A	A	P	P	P	P	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
4	NR	A	A	P	P	P	P	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
8	NR	A	A	P	P	P	P	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
9	NR	A	A	A	P	P	A	NR	P	P	P	P	A	NR	P	P	NR	P	A	A
11	NR	A	A	P	P	P	A	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
14	NR	A	A	P	P	P	A	NR	P	P	P	P	A	NR	P	P	NR	P	A	A
16	NR	P	A	P	P	P	P	NR	P	P	P	P	A	NR	P	P	NR	P	A	P
22	NR	P	A	P	P	P	A	NR	P	P	P	P	A	NR	P	P	NR	P	A	P
109	NR	A	A	P	P	P	P	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
153	NR	A	A	P	P	P	A	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
156	NR	A	A	P	P	P	A	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
157	NR	A	A	P	P	P	A	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
163	NR	A	A	P	P	P	P	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
176	NR	A	A	P	P	P	P	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
178	NR	A	A	P	P	P	P	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
250	NR	A	A	P	P	P	P	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
259	NR	A	A	P	P	P	A	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
262	NR	A	A	P	P	P	P	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
276	NR	A	A	P	P	P	A	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
277	NR	A	A	P	P	P	A	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
278	NR	A	A	P	P	P	A	NR	A	P	P	P	A	NR	P	P	NR	P	A	A

279	NR	A	A	P	P	P	P	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
280	NR	A	A	P	P	P	P	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
320	NR	A	A	P	P	P	P	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
323	NR	A	A	P	P	P	P	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
329	NR	A	A	P	P	P	P	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
336	NR	A	A	P	P	P	P	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
344	NR	A	A	P	P	P	P	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
346	NR	A	A	P	P	P	P	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
347	NR	A	A	P	P	P	P	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
467	NR	A	A	P	P	P	P	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
472	NR	A	A	P	P	P	P	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
475	NR	A	A	P	P	P	P	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
477	NR	A	A	P	P	P	P	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
478	NR	A	A	P	P	P	P	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
497	NR	A	A	P	P	P	P	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
501	NR	A	A	P	P	P	P	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
545	NR	A	A	P	P	P	P	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
546	NR	A	A	P	P	P	P	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
550	NR	A	A	P	P	P	P	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
567	NR	A	A	P	P	P	P	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
682	NR	A	A	P	P	P	P	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
683	NR	A	P	P	P	P	P	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
685	NR	A	A	P	P	P	P	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
686	NR	A	A	P	P	P	P	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
689	NR	A	A	P	P	P	P	NR	A	P	P	P	A	NR	P	P	NR	P	A	A

710	NR	A	A	P	P	P	P	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
713	NR	A	A	P	P	P	P	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
717	NR	A	A	P	P	P	P	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
719	NR	A	A	P	P	P	P	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
729	NR	A	A	P	P	P	P	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
813	NR	A	A	P	P	P	P	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
814	NR	A	A	P	P	P	P	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
815	NR	A	A	P	P	P	A	NR	A	P	P	P	P	NR	P	P	NR	P	A	A
816	NR	A	A	P	P	P	A	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
817	NR	A	A	P	P	P	P	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
818	NR	A	A	P	P	P	P	NR	A	P	P	P	A	NR	P	P	NR	P	A	A
821	NR	A	A	P	P	P	A	NR	A	P	P	P	A	NR	P	P	NR	P	A	A

A: absent, P: present; NR: not retrieved

Table S4. Virulence genes identified per isolate using VirulenceFinder.

	Exoenzymes								Toxins								
Isolate ID	***aur***	***splA***	***splB***	***hlgA***	***hglB***	***hglC***	***lukD***	***lukE***	***sei***	***sem***	***sen***	***seo***	***seu***	***seg***	***tst***	***sel***	***sec***
1	P	P	P	P	P	P	P	P	A	A	A	A	A	A	A	A	A
2	P	P	P	P	P	P	P	P	A	A	A	A	A	A	A	A	A
4	P	A	P	P	P	P	P	P	A	A	A	A	A	A	A	A	A
8	P	P	A	A	P	P	A	P	A	A	A	A	A	A	A	A	A
9	P	A	P	P	P	P	P	P	A	A	A	A	A	A	A	A	A
11	P	P	P	P	P	P	P	A	A	A	A	A	A	A	A	A	A
14	P	P	P	P	P	P	A	A	A	A	A	A	A	A	A	A	A
16	P	P	P	P	P	P	A	A	A	A	A	A	A	A	A	A	A
22	P	P	P	P	P	P	P	P	P	P	P	P	P	P	A	P	A
109	P	P	P	P	P	P	P	P	P	P	P	P	P	P	A	A	A
153	P	P	P	P	P	P	P	P	P	P	P	P	P	P	A	A	A
156	P	P	P	P	P	P	P	P	P	P	P	P	P	P	A	A	A
157	P	P	P	P	P	P	P	P	A	A	A	A	A	A	A	A	A
163	P	P	P	P	P	P	P	P	A	A	A	A	A	A	A	A	A
176	P	P	P	P	P	P	P	P	A	A	A	A	A	A	A	A	A
178	P	P	P	P	P	P	A	A	A	A	A	A	A	A	A	A	A
250	P	P	P	P	P	P	A	A	A	A	A	A	A	A	A	A	A
259	P	P	P	P	P	P	P	A	A	A	A	A	A	A	A	A	A
262	P	P	P	P	P	P	P	P	A	A	A	A	A	A	A	A	A
276	P	P	P	P	P	P	P	A	A	A	A	A	A	A	A	A	A
277	P	P	P	P	P	P	P	P	A	A	A	A	A	A	P	P	P
278	P	A	P	A	P	P	P	P	A	P	A	P	A	P	A	A	A

279	P	P	P	P	P	P	P	P	A	A	A	A	A	A	P	P	P
280	P	P	P	P	P	P	P	P	P	P	P	A	A	P	A	A	A
320	P	P	P	P	P	P	A	A	A	A	A	A	A	A	A	A	A
323	P	P	P	P	P	P	P	P	A	A	A	A	A	A	A	A	A
329	P	P	P	P	P	P	A	A	A	A	A	A	A	A	A	A	A
336	P	P	P	P	P	P	P	A	A	A	A	A	A	A	A	A	A
344	P	P	P	P	P	P	P	P	A	A	A	A	A	A	A	A	A
346	P	P	P	P	P	P	A	A	A	A	A	A	A	A	A	A	A
347	P	P	P	P	P	P	A	A	A	A	A	A	A	A	A	A	A
467	P	P	P	P	P	P	P	A	A	A	A	A	A	A	A	A	A
472	P	P	P	P	P	P	P	P	A	A	A	A	A	A	A	A	A
475	P	P	P	P	P	P	P	P	P	P	P	P	P	A	A	A	A
477	P	P	P	P	P	P	P	P	P	P	P	P	P	P	A	A	A
478	P	P	P	P	P	P	P	P	A	P	P	P	A	P	A	A	A
497	P	P	P	P	P	P	A	A	A	A	A	A	A	A	A	A	A
501	P	P	P	P	P	P	P	P	A	A	A	A	A	A	A	A	A
545	P	P	P	P	P	P	P	A	A	A	A	A	A	A	A	A	A
546	P	P	P	P	P	P	P	P	A	A	A	A	A	A	A	A	A
550	P	P	P	P	P	P	P	P	A	A	P	A	A	A	P	P	P
567	P	A	P	P	P	P	P	P	P	P	P	P	P	P	P	P	A
682	P	P	P	P	P	P	P	P	A	A	A	A	A	A	A	A	A
683	P	A	A	P	P	P	P	P	A	A	A	A	A	A	A	A	A
685	P	P	P	P	P	P	A	A	A	A	A	A	A	A	A	A	A
686	P	A	P	P	P	P	P	A	A	A	A	A	A	A	A	A	A
689	P	A	A	P	P	P	P	P	A	A	A	A	A	A	A	A	A

710	P	P	P	P	P	P	P	P	A	A	A	A	A	A	A	A	A
713	P	P	P	P	P	P	P	A	A	A	A	A	A	A	A	A	A
717	P	A	P	P	P	P	A	A	A	A	A	A	A	A	A	A	A
719	P	A	P	P	P	P	P	P	P	P	P	P	P	A	A	A	A
729	P	P	P	P	P	P	A	A	A	A	A	A	A	A	A	A	A
813	P	P	P	P	P	P	P	P	A	A	A	A	A	A	A	A	A
814	P	P	P	P	P	P	P	P	P	P	P	P	P	A	A	A	A
815	P	P	P	P	P	P	P	P	P	P	P	P	P	A	A	A	A
816	P	A	A	P	P	P	P	P	A	A	A	A	A	A	A	A	A
817	P	A	A	P	P	P	P	P	A	A	A	A	A	A	A	A	A
818	P	A	A	P	P	P	P	P	A	A	A	A	A	A	A	A	A
821	P	A	A	P	P	P	P	P	A	A	A	A	A	A	A	A	A

A: absent; P: present.

Table S5. Reference genomes used for phylogenetic tree (information retrieved from PATRIC).

Genome ID	Name	Origin	Isolation Source	ST
561307.5	*Staphylococcus aureus* subsp. *aureus* RN4220	Human	Not specified	ST8
985006.4	*Staphylococcus aureus* subsp. *aureus* LGA251 (MRSA)	Bovine	Milk	ST425
1343064.3	*Staphylococcus aureus* subsp. *aureus* ST772-MRSA-V	Human	Clinical isolate	ST722
523796.5	Staphylococcus aureus subsp. *aureus* ST398 strain S0385	Human	Endocarditis	ST398
273036.6	*Staphylococcus aureus* RF122	Human	Mastitis	ST151
1280.11362	*Staphylococcus aureus* strain pig swab (LA-MRSA)	Pig	Farm	ST398
1280.10463	*Staphylococcus aureus* strain type strain: MSSA120	Human	Wound	ST8
158879.11	*Staphylococcus* aureus subsp. *aureus* N315	Human	Pharyngeal smear	ST5
1280.16546	*Staphylococcus aureus* strain MOK063	Bovine	Milk	ST97
1280.5751	*Staphylococcus aureus* strain st133	Human	Not specified	ST22
1388109.3	*Staphylococcus aureus* M0168	Human	Nares	ST105
1280.10472	*Staphylococcus aureus* strain MSSAT120	Human	Wound	ST8
1116218.3	*Staphylococcus aureus* subsp. *aureus* VRS5	Human	Clinical isolate	ST8
46170.363	*Staphylococcus aureus* subsp. *aureus* strain TW20	Human	Bacteraemia	ST238
1280.10409	*Staphylococcus aureus* strain MRSA073B	Human	Nares	ST8
1182756.4	*Staphylococcus aureus* subsp. *aureus* str. Newbould 305	Bovine	Mastitis	ST115
1280.16547	*Staphylococcus aureus* strain MOK042	Bovine	Milk	ST71
426430.24	*Staphylococcus aureus* subsp. *aureus* str. Newman	Human	Invasive infection	ST8
1280.11678	*Staphylococcus aureus* strain USA300-SUR12	Human	Wound	ST8

Table S6. Tree analysis statistics.

Number of genomes	78
Maximum Allowed Deletions	0
Maximum Allowed Duplications	0
Single-copy genes requested	100
Single-copy genes found	92
Number of protein alignments	92
Number of aligned amino acids	15438
Number of CDS alignments	78
Number of aligned nucleotides	37539
RAxML Version	8.2.11

Table S7. Gene families used for the phylogenetic tree construction ranked by alignment, score combining mean per-position variability and alignment length.

PGFam	Align. Score	Align. Length	Num Seqs	Mean Sqr Freq	Used In Analysis	Product
PGF_00009966	21.40	463	76	0.994	True	Glycyl-tRNA synthetase (EC 6.1.1.14)
PGF_00057483	20.79	443	76	0.988	True	Transcription termination factor Rho
PGF_01915472	20.63	430	76	0.995	True	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex (EC 2.3.1.12)
PGF_03469467	19.82	414	76	0.974	True	TPR-repeat-containing protein
PGF_00050641	18.71	351	76	0.999	True	Sensor histidine protein kinase SaeS (EC 2.7.13.3) (exoprotein expression protein S)
PGF_00051970	18.15	330	76	0.999	True	Siderophore staphylobactin ABC transporter, substrate-binding protein SirA
PGF_00005688	17.77	354	76	0.945	True	Flavin-utilizing monoxygenase
PGF_00420953	17.64	313	76	0.997	True	3-oxoacyl-[acyl-carrier-protein] synthase, KASIII (EC 2.3.1.180)
PGF_00019248	17.55	309	76	0.999	True	Manganese-dependent inorganic pyrophosphatase (EC 3.6.1.1)
PGF_09201380	17.39	324	76	0.966	True	DNA polymerase III delta subunit (EC 2.7.7.7)
PGF_07157721	17.38	303	76	0.999	True	Heme O synthase, protoheme IX farnesyltransferase, COX10-CtaB
PGF_00018090	17.02	291	76	0.997	True	LytH protein involved in methicillin resistance / N-acetylmuramoyl-L-alanine amidase (EC 3.5.1.28) domain
PGF_06032469	16.81	285	76	0.996	True	FIG002434: Uncharacterized protein YtpQ
PGF_00057413	16.78	283	76	0.998	True	Transcription antiterminator
PGF_02905288	16.55	274	76	1.000	True	FIG002540: Haloacid dehalogenase-like hydrolase
PGF_00425662	16.37	268	76	1.000	True	FIG00002411: Thioredoxin-fold protein
PGF_02280759	15.96	256	76	0.997	True	Undecaprenyl diphosphate synthase (EC 2.5.1.31)
PGF_00847718	15.72	261	76	0.973	True	Aliphatic amidase AmiE (EC 3.5.1.4)
PGF_07097873	15.61	382	76	0.799	True	Ribonuclease Z (EC 3.1.26.11)
PGF_00054142	15.43	241	76	0.994	True	Staphylococcal respiratory response protein SrrA
PGF_10565701	15.21	314	76	0.859	True	ABC transporter, permease protein 2 (cluster 5, nickel/peptides/opines)

PGF_00058741	15.11	237	76	0.981	True	Transcriptional regulator in cluster with Zn-dependent hydrolase
PGF_00011471	14.96	228	76	0.991	True	Hemolysin III
PGF_08228852	14.93	280	76	0.893	True	Zinc ABC transporter, ATP-binding protein ZnuC
PGF_00416313	14.81	254	76	0.929	True	Capsular polysaccharide synthesis enzyme Cap5C; Manganese-dependent protein-tyrosine phosphatase (EC 3.1.3.48)
PGF_06780452	14.42	215	76	0.984	True	Multidrug resistance protein [function not yet clear]
PGF_01756668	14.32	205	76	1.000	True	UPF0111 protein YkaA, likely to be a phosphate transport regulator
PGF_00013206	14.07	199	76	0.997	True	Hypothetical protein SAV1854
PGF_00041794	13.75	189	76	1.000	True	Alkyl hydroperoxide reductase protein C (EC 1.11.1.15)
PGF_03789030	13.71	188	76	1.000	True	Protein-arginine kinase activator protein McsA
PGF_00045787	13.61	186	76	0.998	True	Pyridoxal 5'-phosphate synthase (glutamine hydrolyzing), glutaminase subunit (EC 4.3.3.6)
PGF_03091958	13.57	191	76	0.982	True	hypothetical protein
PGF_00048926	13.55	184	76	0.999	True	Ribosome recycling factor
PGF_03475527	13.26	176	76	0.999	True	Hypothetical protein SAV1896
PGF_00013251	13.25	176	76	0.999	True	Hypothetical protein SAV2322
PGF_00072447	13.22	178	76	0.991	True	hypothetical fig|282458.1.peg.578 homolog
PGF_00418664	12.99	173	76	0.988	True	Colicin V production protein
PGF_10545981	12.82	195	76	0.918	True	hypothetical protein
PGF_00007489	12.76	163	76	1.000	True	UPF0478 protein YtxG
PGF_02008717	12.64	236	76	0.823	True	Late competence protein ComC, processing protease
PGF_00486077	12.44	157	76	0.993	True	hypothetical protein
PGF_00020361	12.34	155	76	0.991	True	Metal-dependent hydrolase YbeY, involved in rRNA and/or ribosome maturation and assembly
PGF_05697437	12.33	180	76	0.919	True	Alkaline shock protein 23
PGF_00413872	12.31	159	76	0.976	True	transmembrane protein, distant homology with ydbS
PGF_00012657	12.31	152	76	0.998	True	ACT domain-containing protein
PGF_03089396	12.28	151	76	0.999	True	Transcriptional regulator, MarR family
PGF_05117700	12.22	154	76	0.985	True	Free methionine-(R)-sulfoxide reductase, contains GAF domain

PGF_05977531	12.03	151	76	0.979	True	hypothetical protein
PGF_01151115	11.95	147	76	0.986	True	Antiholin-like protein LrgA
PGF_02139563	11.75	139	76	0.996	True	6-carboxy-5,6,7,8-tetrahydropterin synthase (EC 4.1.2.50)
PGF_00064587	11.51	160	76	0.910	True	Uncharacterized N-acetyltransferase BT9727_3663 (EC 2.3.1.-)
PGF_10324033	11.40	130	76	1.000	True	Cell division protein DivIC (FtsB), stabilizes FtsL against RasP cleavage
PGF_04713899	11.31	128	76	1.000	True	Rhodanese-like domain protein
PGF_00004239	11.05	122	76	1.000	True	FemC, factor involved in methicillin resistance / Glutamine synthetase repressor
PGF_00072445	10.98	165	76	0.855	True	hypothetical fig|282458.1.peg.572 homolog
PGF_00301663	10.98	125	76	0.982	True	hypothetical protein
PGF_04791864	10.98	121	76	0.998	True	hypothetical protein
PGF_12836853	10.80	122	76	0.978	True	hypothetical protein
PGF_00435430	10.66	117	76	0.986	True	Ribosomal silencing factor RsfA
PGF_03681316	10.61	121	76	0.965	True	Hemoglobin-like protein HbO
PGF_06632167	10.48	110	76	0.999	True	Signal recognition particle associated protein
PGF_00499795	10.34	108	76	0.995	True	Anti-sigma B factor antagonist RsbV
PGF_00067599	10.26	106	76	0.997	True	Zn(II) or Co(II)-specific transcriptional repressor protein
PGF_00417618	10.24	105	76	0.999	True	Chitinase B
PGF_01934082	10.16	106	76	0.987	True	Uncharacterized protein YtxJ
PGF_00417093	10.15	125	76	0.907	True	Cell division protein GpsB, coordinates the switch between cylindrical and septal cell wall synthesis by re-localization of PBP1
PGF_00013169	10.10	104	76	0.991	True	Hypothetical protein MW1689
PGF_00422085	9.99	100	76	0.999	True	DNA-binding protein SpoVG
PGF_07836795	9.98	100	76	0.998	True	Aspartyl-tRNA(Asn) amidotransferase subunit C (EC 6.3.5.6) @ Glutamyl-tRNA(Gln) amidotransferase subunit C (EC 6.3.5.7)
PGF_00057451	9.96	104	76	0.976	True	Transcription regulator CDS_ID OB0894
PGF_09452281	9.96	130	76	0.873	True	Ribosome-binding factor A
PGF_00411944	9.95	105	76	0.971	True	Ribosomal protein L7Ae family protein YlxQ
PGF_00066218	9.91	100	76	0.991	True	Urease gamma subunit (EC 3.5.1.5)
PGF_00056802	9.85	106	76	0.956	True	Thioredoxin-like protein YdbP
PGF_00827526	9.83	97	76	0.998	True	6 kDa early secretory antigenic target ESAT-6 (EsxA)
PGF_00420218	9.72	96	76	0.992	True	Cytochrome aa3-600 menaquinol oxidase subunit IV

PGF_12692517	9.70	95	76	0.995	True	hypothetical protein
PGF_03081665	9.70	96	76	0.990	True	Cell division integral membrane protein, YggT and half-length relatives
PGF_00001347	9.51	91	76	0.997	True	FIG010063: hypothetical protein
PGF_00419414	9.50	91	76	0.996	True	UPF0358 protein YlaN
PGF_01175502	9.49	90	76	1.000	True	DNA-binding protein HBsu
PGF_00013347	9.27	86	76	1.000	True	UPF0297 protein YrzL
PGF_02454577	9.11	83	76	1.000	True	SSU ribosomal protein S20p
PGF_00043859	8.93	80	76	0.998	True	Putative secretion accessory protein EsaB/YukD
PGF_10505094	8.74	77	76	0.997	True	Uncharacterized membrane protein YwzB
PGF_00046622	8.73	77	76	0.995	True	RNA-binding protein Hfq
PGF_00308739	8.71	126	76	0.776	True	hypothetical protein
PGF_00013230	8.71	79	76	0.980	True	Hypothetical protein SAV2183
PGF_00002343	8.60	78	76	0.973	True	DUF1450 superfamily protein
PGF_00064156	8.45	74	76	0.982	True	UPF0033 protein YeeD
PGF_12745086	8.44	189	76	0.614	True	Uncharacterized protein SufA
PGF_07706058	8.37	72	76	0.986	True	hypothetical protein
PGF_02705782	8.19	68	76	0.994	True	Copper(I) chaperone CopZ
PGF_00013249	7.81	61	76	1.000	True	Hypothetical protein SAV2318
PGF_00037476	7.46	56	76	0.996	True	Programmed cell death antitoxin YdcD
PGF_00128973	7.42	55	76	1.000	True	hypothetical protein
PGF_00198850	7.40	57	76	0.980	True	Uncharacterized protein YebG
PGF_00013157	7.27	53	76	0.999	True	Hypothetical protein FIG009695
PGF_03062309	7.09	66	76	0.873	True	hypothetical protein
PGF_00299296	7.07	50	76	0.999	True	Component involved in D-alanylation of teichoic acids
PGF_00013213	6.71	45	76	1.000	False	Hypothetical protein SAV2085
PGF_03469120	6.08	38	76	0.986	False	Hypothetical protein

CHAPTER V – CONCLUSIONS AND PERSPECTIVE

CONCLUSIONS AND PERSPECTIVE

As stated throughout this thesis, the emergence of multidrug resistant bacteria together with the limited effective antibacterial therapeutic options, is a major public health concern and highlights the need to develop novel antimicrobial therapies, in particular to combat multidrug resistant Gram-negative related infections. Repurposing of existing drugs beyond its original application, poses as an attractive strategy for exploiting novel therapies and, at the same time, to reduce costs and development time associated with the drug discovery process [58,96,179,180].

Multiple studies focus on repurposing already existing and FDA approved drugs to treat bacterial infections [112,113,181] and, in the past years, there has been a growing interest in repurposing niclosamide, due to its broad range of biological activities and clinical applicability [132,133,142,143,134–141]. Niclosamide potential is further translated in the number of related publications that can be found on PubMed. In total one can found 1022 publications since its discovery up to December 2019 (Figure 8). Nevertheless, only a small number of these publications cover the repurposing of niclosamide for the treatment of infectious diseases, specifically those instigated by bacteria. The potential of niclosamide to be repurposed as an antibacterial agent and its effectiveness towards *S. aureus* and other bacteria was already reported [132,136].

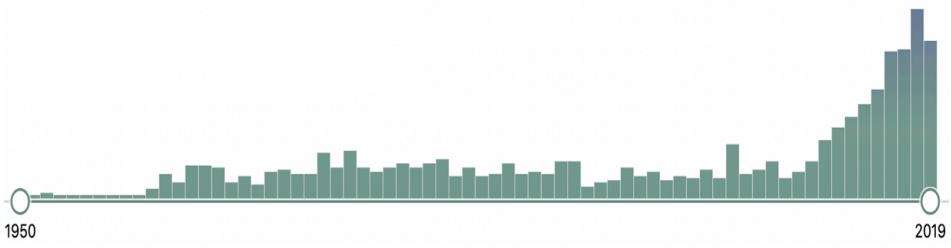

Figure 8. Distribution of niclosamide related publications since its discovery in 1950 up to 2019 (data recovered from PubMed on December 2019)

Antibiotic options directed to treat *S. aureus* load in atopic dermatitis skin lesions are becoming scarce due to displayed resistance [148]. Here we described the potential of ATx201 (niclosamide)

as a potent *S. aureus* decolonizing agent. In addition to its narrow MIC distribution towards *S. aureus* and low probability to develop resistance *in vitro*, during the clinical trials ATx201 was shown to reduce *S. aureus* bacterial load in atopic dermatitis lesions without significantly affecting the skin commensal microbiota. Altogether, this suggests that ATx201 could potentially be a novel treatment for atopic dermatitis by overcoming resistance issues related with current therapies. We described, as well, with further detail ATx201 mode of action towards Gram-positive bacteria, in specific *S. aureus*, adding to the knowledge available regarding how niclosamide exerts its antimicrobial action (Manuscript I).

Although a very promising compound, ATx201 lacks activity towards Gram-negative bacteria, with exception to *H. pylori* [136]. Multiple SAR (structure-activity relationship) studies reported improvement in salicylanilides compounds activity and other pharmacological traits (e.g. toxicity) [134,145,147,182,183], nevertheless potentiation of these compounds towards Gram-negative bacteria remains a challenge. In Manuscript II, we evaluated ATx201 and novel derivative compounds antimicrobial potency towards multidrug resistant bacteria, including Gram-negative bacteria. Albeit at high concentrations, several compounds were identified as having activity towards this group of bacteria. Though their clinical applicability might be low, screening data relative to these compounds contributes to deepen the knowledge about the class and provides useful information, enabling further optimization. In addition, we described the possible role of tolC in the lack of activity displayed by ATx201 and ATx201 potentiation through drug combination, reinforcing ATx201 and derivatives potential to treat Gram-negative related infections.

These studies generated important data regarding novel antimicrobial compounds profile. Nevertheless, given ATx201 and derivative compounds antimicrobial potential and the possibility to use these compounds in the future to treat infectious diseases, besides characterizing compounds safety profile, more research focusing on ATx201 and in particular the derivative compounds mode of action and how this may differ from the one observed for ATx201, are necessary. As well, studies to understand the mechanism behind ATx201 and UNIP01 synergistic

activity are required, since it can further contribute to compound optimization and to understand the compounds full therapeutic potential.

In Manuscript III, the focus was the epidemiological characterization of *S. aureus* recovered from Danish dairy cows. Understanding bacteria epidemiology with respect to prevalence, dominant lineages and trends in clonal dissemination, can help to control the spread of antibiotic resistance and the transmission of pathogenic determinants across different hosts and niches [29,45].

We analyzed *S. aureus* resistance profile both from a phenotypic and genotypic perspectives. High rates of phenotypic resistance were observed, which was not expected in relation to previous studies carried out in Denmark [184,185]. Additionally, we noted discrepancies between observed phenotype and expected genotypic profile. While more studies are necessary to assess the reasons and mechanisms behind the high levels of phenotypic resistance observed among these isolates, this study enhances the consequences of the continuous use of antibiotics over the years.

Currently, there are very few antibiotics in late-phase clinical trials that are expected to reach the market, as well many of these antibiotics are derivatives of already existing classes. In due time this can become a problem, since there is a higher probability that bacteria develop resistance at a faster pace [58,87,90]. Given this scenario, besides the development of novel antibiotic therapies it is important to understand patterns of antibiotic resistance and possible factors and mechanisms contributing to resistance. Likewise, understanding discrepancies between resistance phenotypes and genotypes, is essential and, it might aid the identification of novel mechanisms of resistance and potential novel antimicrobial targets. Altogether, this information can contribute to improve existing therapies (e.g. through drug combination) or to develop novel ones.

There is no doubt that antibiotic resistance is a problem of great concern that will continue to increase in lack of a continuous investment in antibiotic drug research and development pipelines combined with stronger regulations towards antibiotic use, both in the clinic and veterinary practices.

In conclusion, the search for new antimicrobial compounds is crucial to overcome antibiotic resistance and many lessons can be retrieved from past discovery and development efforts. Combining that knowledge with antibiotic resistance patterns and awareness on the multiple mechanisms that allow bacteria to acquire and develop resistance is likely to expand our understanding on the biological events underlying antibiotic resistance and hopefully, deliver novel antibiotic therapies.

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