A METHOD OF TREATMENT OF A BACTERIAL INFECTION

FIELD OF THE INVENTION

The present invention relates to the field of medicine, specifically the field of bacterial infection and treatment thereof.

BACKGROUND OF THE INVENTION

The human pathogen *Staphylococcus aureus* colonizes approximately a third of all humans and is one of the leading causes of bacteremia and infective endocarditis in the industrialized world (1). In addition to emerging antibiotic resistance, persisting and recurrent infections substantially add to morbidity and mortality (2,3). Recurrence rates, in particular after osteomyelitis or endocarditis, are high and infections may relapse even years after apparent cure (4). Infection recurrence is associated with SCVs (small colony variants) and/or non-replicating persisters for several reasons (5-8). Their arrested or slow growth and reduced metabolism renders antibiotics inefficient (9-13). Moreover, they preferentially hide in privileged locations such as in abscesses and within host cells (14-16). These privileged locations shield them from the host's innate immune system and from extracellularly active antibiotics. In addition, antibiotics do not penetrate abscesses efficiently and are less active due to the low pH (17-21). Therefore the 'ubi pus ibi evacua' – the necessity of surgical removal of abscesses formulated in the antiquity still applies nowadays despite highly active antibiotics. In contrast to abscesses, intracellular bacteria cannot be mechanically removed and often resist eradication by currently available antibiotics. Most SCVs isolated in clinics revert to the large colony phenotype upon sub-cultivation. Due to these unstable properties, stable genetically-modified SCV mutants with defects in the electron transport system have been used to find new strategies to target SCVs and have been found to localize in host cell lysosomes (22). However, stable SCVs only partially reflect the clinical SCVs. They are less virulent (23) and do not revert to the highly virulent and fast-growing phenotype. Consequently, there is an urgent need for means to study clinical SCVs and persisters and ultimately an effective method of treatment of SCVs and persisters.

SUMMARY

In one aspect of the present disclosure, there is provided the use of an effective amount of an agent that increases the intracellular pH of a host cell and/or of an intracellular compartment of a host cell and an effective amount of a bactericidal agent in the manufacture of a medicament for the treatment of a bacterial infection in a subject in need thereof.

In another aspect, there provided a chimeric bactericidal polypeptide comprising a functional enzymatic domain from a cell wall lytic enzyme selected from the group consisting of SEQ ID NO: 1 - 7, or a variant thereof and a protein transduction domain selected from the group consisting of SEQ ID NO: 12 - 25, or a variant thereof and optionally comprising a cell wall binding domain is selected from the group consisting of SEQ ID NO: 8 - 11, or a variant thereof and/or an antimicrobial peptide selected from the group consisting of SEQ ID NO: 70 - 90, or a variant thereof.

In another aspect, there is provided a polynucleotide encoding the chimeric bactericidal polypeptide as disclosed above.

DETAILED DESCRIPTION OF THE INVENTION

Surprisingly, the inventors have established that reversible persisters and reversible SCV's can be induced by low pH and that low pH adapted bacteria persisted intracellularly, specifically within lysosomes (low pH organelles). Low pH induced SCVs and persisters reverted to the highly virulent phenotype upon raising the pH. Raising the pH intracellularly reverted the low pH adapted bacteria *in vitro* and *in vivo* and rendered the SCV's and persisters susceptible to antibacterial agents that are usually not effective intracellularly. This provide a completely new way of preventing and/or treating infections that persist primarily or at least partly intracellularly in intracellular compartments, such as but not limited to infections of the skin, of the respiratory or tonsillar epithelium (pharyngotonsillitis), (bovine) mastitis, or eradicating vaginal *S. agalactiae* infection (in macrophages) in pregnant women to prevent neonatal sepsis or preventing/treating *S. pneumoniae*–induced monocyte–derived macrophage apoptosis, or treating tuberculosis, leprosy, listeriosis, typhoid fever, bacillary dysenteria, plague, brucellosis, typhus, Rocky Mountains spotted fever, chlamydia, trachoma..

Accordingly, in a first aspect the invention provides for a method of treatment of a bacterial infection in a subject in need thereof, comprising:

- administration of an effective amount of an agent that increases the intracellular pH of a host cell and/or of an intracellular compartment of a host cell, and
- administration of an effective amount of a bactericidal agent. Said method is herein referred to as a method according to the invention.

Preferably, in the embodiments of the invention, the increase in pH activates a non-replicating intracellular bacterium and the bactericidal agent kills the activated intracellular bacterium.

In the embodiments of the invention, the bacterial infection is preferably a persistent bacterial infection and may be related to a persistent *S. aureus* infection of various tissues such as but not limited to skin, respiratory epithelium, tonsillar tissue, mastitis. In the embodiments of the invention, the bacterial infection is preferably an infection by a species selected from the group bacteria consisting of Staphylococcus, Streptococcus (such as *S. pyogenes*, *S. agalactiae* and *S.pneumonia*), Actinomyces, Nocardia, Bacillus (such as *B. anthracis*), Coxiella (such as *Coxiella burnetii*), Rickettsia, Mycobacteria (such as *M. tuberculosis* and *M. leprae*), Legionella (such as *L. pneumophila*), Mycoplasma, Salmonella (such as *S. typhimurium*), Shigella (such as *S. dysenteriae*),

Yersinia (such as *Y. pestis*), Brucella, Listeria (such as *L. monocytogenesis*), Actinobacillus (such as *A. actinomycetemcomitans*), Gardnerella (such as *G. vaginalis*), Chlamydia (such as *C. trachomatis*), and Chlamidophila; a more preferred bacterium is a species of Staphylococcus; a preferred species of Staphylococcus is *S. aureus*.

In the embodiments of the invention, a bacterium causing the bacterial infection is preferably present intracellularly in a host cell, such as in the cytosol; more preferably a bacterium causing the bacterial infection is preferably present within an intracellular compartment *S. aureus* Preferably, the bacterial infection comprises a population of bacteria comprising at least one subpopulation of bacteria that is resistant to a bactericidal agent (e.g. an antibiotic), such as, but not limited to MRSA. In the embodiments of the invention, the intracellular compartment may be any intracellular compartment wherein a bacterium can be persistently present and/or persistently present; preferably the intracellular compartment is a compartment with low pH. A preferred intracellular compartment is a compartment selected from the group consisting of an endosome, a lysozome, a phagosome, a phagolysosome, an autophagosome and an autolysosome'a more preferred intracellular compartment is a phagosome, lysozome of phagolysosome; an even more preferred intracellular compartment is a phagolysosome..

In the embodiments of the invention within a subject in need of treatment, the infected host cell is preferably a eukaryotic host cell; the eukaryotic host cell is preferably selected from the group consisting of professional and non-professional phagocytes, a lymphoid cell, a tonsillar tissue cell, a respiratory epithelial cell, a buccal epithelial cell, a bone marrow cell, an osteoblast, a keratinocyte, a muscle cell, a monocyte, a macrophage, a dendritic cell, an endothelial cell, an epithelial cell, a fibroblasts, an astrocyte, and an microglial cell; a more preferred eukaryotic host cell is a professional or a non-professional phagocyte. The subject may be any subject susceptible to or suffering from a persistent bacterial infection, such as, but not limited to, a human being or an animal. The animal may be a domestic animal or may be a breeding animal, pet, cattle, poultry or the like. A preferred subject is a human being and may be a newborn, a juvenile, an adult or an aged subject.

In the embodiments of the invention, the agent that increases the intracellular pH and/or the pH of the intracellular compartment may be any agent known to the person skilled in the art that effectively increases the intracellular pH and/or the pH of the intracellular compartment.

Preferably, in the embodiments of the invention, the agent that increases the intracellular pH and/or the pH of the intracellular compartment is an alkalizing agent, preferably an lysosomotropic alkalizing agent, preferably selected from the group of chloroquine, bafilomycin A1, ammonium chloride; a more preferred alkalizing agent is chloroquine. A preferred alkalyzing agent is one that is capable of entering the host cell and more preferably is targeted to the intracellular compartment wherein the infecting bacterium is present. The alkalizing agent may passively enter the cell or a vehicle may be used. The vehicle may be any vehicle known to a person skilled in the art that enables delivery into a host cell.

The term "low pH" in the embodiments of the invention is defined as a pH that is lower than pH 7.4; preferably low pH is about pH 7.0, 6.8, 6.6, 6.4, 6.2, 6.0, 5.8, 5.6, 5.4, 5.2, 5.0, 4.8, 4.6, 4.4, 4.2, 4.0, 3.8, 3.6, 3.4, 3.2, 3.0 or about 2.8. A low pH is preferably a pH lower than about 6.5 A more preferred low pH is about 5.0.

In the embodiments of the present invention, the term raising the pH and increasing the pH are used interchangeably and are defined as raising the pH about 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, 2.2, 2.4, 2.6, 2.8, 3.0, 3.2, 3.4, 3.6, 3.8, 4.0, 4.2, 4.4, 4.6 or about 4.8. Preferably, the pH is raised to within the range of about pH 6 to about7; preferably to within the range of about 6.5 to about 7

The term "an effective amount" in the embodiments of the invention in the context of an agent that increases the intracellular pH and/or the pH of an intracellular compartment is defined as any amount that is sufficient to raise the intracellular pH and/or the pH of an intracellular compartment at least about 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, 2.2, 2.4, 2.6, 2.8, 3.0 to reach the range as depicted here above.. The exact amount used will *inter alia* depend on the agent used and on the bodyweight of the subject.

The term "an effective amount" in the embodiments of the invention in the context of a bactericidal agent is defined as any amount that is sufficient to have a bactericidal affect intracellularly and/or within the intracellular compartment. The exact amount used will *inter alia* depend on the agent used and on the bodyweight of the subject.

Intracellular pH and the pH of an intracellular compartment can be assessed by any means known to the person skilled in the art. In the embodiments of the invention, the bactericidal agent may be any bactericidal agent known to the person skilled in the art and preferably is a bactericidal agent capable of entering the host cell and/or the intracellular compartment of the host cell. A preferred bactericidal agent is a chimeric

bactericidal agent. A preferred bactericidal agent is selected from the group consisting of a bacteriocin or a functional part thereof, a bacterial lysin or autolysin or a functional part thereof, a bacteriophage lysin or a functional part thereof, a viral lysin or a part thereof, an antimicrobial peptide and an antibiotic. A further preferred bactericidal agent is a bacteriophage derived lytic structural protein (such as a tail lysin and a virion associated lytic protein) or an isolated lytic domain from such lytic structural protein or bacteriophage lysin. The bactericidal agents of the invention may be used alone or may be used in combinations of two or three or more bactericidal agents of the invention. A preferred bactericidal agent is one that is capable of entering the host cell and more preferably is targeted to the intracellular compartment wherein the infecting bacterium is present. The bactericidal agent may passively enter the cell or a vehicle may be used. The vehicle may be any vehicle known to a person skilled in the art that enables delivery into a host cell.

The antibiotic may be any antibiotic known to the person skilled in the art. A preferred antibiotic is selected from the group consisting of beta-lactam antibiotics such as penicillin derivatives, cephalosporins, monobactams, carbapenems, vancomycins, daptomycin, fluoroquinolones, metronidazole, nitrofurantoin, co-trimoxazole, telithromycin, aminoglycosidic antibiotics; a more preferred antibiotic is flucloxacillin.

The bacteriocin may be any bacteriocin known to the person skilled in the art, preferably a bacteriocin of any Class I –IV.

Class I bacteriocins herein are small peptide inhibitors and include nisin and other lantibiotics.

Class II bacteriocins herein are small (<10 kDa) heat-stable proteins. This class is subdivided into five subclassses. The class IIa bacteriocins (pediocin-like bacteriocins) are the largest subgroup and contain an N-terminal consensus sequence -Tyr-Gly-Asn-Gly-Val-Xaa-Cys across this group. The C-terminal is responsible for species-specific activity, causing cell-leakage by permeabilizing the target cell wall. The class IIb bacteriocins (two-peptide bacteriocins) require two different peptides for activity. One such an example is lactococcin G, which permeabilizes cell membranes for monovalent ions such as Na and K, but not for divalents ones. Almost all of these bacteriocins have a GxxxG motif. This motif is also found in transmembrane proteins where they are involved in helix-helix interactions. The bacteriocin's GxxxG motif can interact with the motifs in the membranes of the bacterial cells and kill the bacteria by doing so. Class IIc

encompasses cyclic peptides, which possesses the N-terminal and C-terminal regions covalentely linked. Enterocin AS-48 is the prototype of this group. Class IId cover single-peptide bacteriocins, which are not post-translated modified and do not show the pediocin-like signature. The best example of this group is the highly stable aureocin A53. This bacteriocin is stable under highly acidic environment (HCl 6 N), not affected by proteases and thermoresistant. The most recently proposed subclass is the Class IIe, which encompasses those bacteriocins composed by three or four non-pediocin like peptides. The best example is aureocin A70, a four-peptides bacteriocin, highly active against *L. monocytogenes*, with potential biotechnological applications.

Class III bacteriocins are large, heat-labile (>10 kDa) protein bacteriocins. This class is subdivided in two subclasses: subclass IIIa or bacteriolysins and subclass IIIb. Subclass IIIa comprises those peptides that kill bacterial cells by cell-wall degradation, thus causing cell lysis. The best studied bacteriolysin is lysostaphin, a 27 kDa peptide that hydrolises several *Staphylococcus* spp. cell walls, principally *S. aureus*. Subclass IIIb, in contrast, comprises those peptides that do not cause cell lysis, killing the target cells by disrupting the membrane potential, which causes ATP efflux .

Class IV bacteriocins are defined as complex bacteriocins containing lipid or carbohydrate moities. Confirmatory experimental data was only recently established with the characterisation of Sublancin and Glycocin F (GccF) by two independent groups.

A preferred bacteriocin is selected from the group consisting of an acidocin, actagardine, agrocin, alveicin, aureocin, aureocin A53, aureocin A70, carnocin, carnocyclin circularin A, colicin, Curvaticin, divercin, duramycin, Enterocin, enterolysin, epidermin/gallidermin, erwiniocin, gassericin A, glycinecin, halocin, haloduracin, lactocin S, lactococin, lacticin, leucoccin, lysostaphin macedocin, mersacidin, mesentericin, microbisporicin, microcin S, mutacin, nisin, paenibacillin, planosporicin, pediocin, pentocin, plantaricin, pyocin, reutericin 6, sakacin, salivaricin, subtilin, sulfolobicin, thuricin 17, trifolitoxin, variacin, vibriocin, warnericin and a warnerin.

The bacteriocin may be from a bacterium itself (24), such as, but not limited to a pyocin from *Pseudomonas aeruginosa*, preferably pyocin SA189 (25).

The antimicrobial peptide may be any antimicrobial peptide known to the person skilled in the art. Sometimes in the art, antimicrobial peptides are considered bacteriocins as listed here above. A preferred antimicrobial peptide is selected from the group consisting

of a cationic or polycationic peptide, an amphipatic peptide, a sushi peptide, a defensin and a hydrophobic peptide.

The bacterial autolysin may be any a bacterial autolysin known to the persons killed in the art. A preferred bacterial autolysin is LytM.

The bacteriophage lysin may be any bacteriophage lysin known to the persons skilled in the art. Herein, the terms bacteriophage lysin, bacteriophage endolysin and endolysin are used interchangeably. A preferred endolysin is selected from the group defined in WO2012/150858, in WO2013/169104, in WO2011/023702, and in WO2012146738, which are herein incorporated by reference with their entire content.

The bactericidal agent may be any agent described here above or be a functional fragment thereof. Herein, the term functional fragment is interchangeably used with the term functional domain. A functional fragment is herein defined as a fragment that still has at least 20, 30, 40, 50, 60, 70, 80, 90, 95, or 99, or at least or 99.9% bactericidal activity as compared to the parent wherefrom the functional fragment is derived when assayed in identical conditions. Preferred functional fragments of bactericidal agents are described in WO2012/150858 and in WO2013/169104.

The bactericidal agent may be a fusion of a bactericidal agent and a functional fragment of bactericidal agents or may be a fusion of different, similar or identical bactericidal agents or of functional fragments of bactericidal agents.

The inventors have come to the surprising finding that the efficiency of a method of treatment according to the invention is greatly enhance when the bactericidal agent is targeted into the host cell. Such targeting may be achieved any means known to the person skilled in the art, as already depicted here above. A preferred means is a protein transduction domain that is operably linked to the bactericidal agent; further referred to herein as a protein transduction domain according to the invention. The term “protein transduction domain” is herein interchangeably used with the term “cell permeable protein (CPP)” and with the term “membrane translocating sequence”. Operably linked is defined herein as such association of the protein transduction domain with the bactericidal agent that the bactericidal agent is targeted into the cell. A preferred operable linkage is fusion by means of covalent binding of the protein transduction domain to the bactericidal agent.

In the embodiments of the invention, the bactericidal agent preferably comprises a functional enzymatic domain of a cell wall lytic enzyme. A cell wall lytic enzyme is

herein defined as any bactericidal agent that acts upon the cell wall of the bacterium it is effective against.

Preferably in the embodiments of the present invention, the bactericidal agent comprises a functional domain from a cell wall lytic enzyme and further comprises a protein transduction domain according to the invention. Preferably in the embodiments according to the invention, the bactericidal agent may further comprise an antimicrobial peptide. Such fusion bactericidal agent preferably is a cell wall lytic enzyme fused to an antimicrobial peptide selected from the group consisting of a cationic or polycationic peptide, an amphipatic peptide, a sushi peptide, a defensin and a hydrophobic peptide, more preferably a fusion protein as defined in US8,383,102 which is herein incorporated by reference in its entirety. Such bactericidal agent fused to an antimicrobial peptide may further comprise a protein transduction domain according to the invention.

In the embodiments of the invention, the bactericidal agent as described here above comprising a functional enzymatic domain of a cell wall lytic enzyme and/or comprising a protein transduction domain and/or comprising an antimicrobial peptide, may further preferably further comprises a cell wall binding domain. Said cell wall binding domain preferably is a cell wall binding domain that binds the peptidoglycan cell wall of the bacteria causing the bacterial infection to be treated.

In the embodiments of the invention, the protein transduction domain may be any such domain known to the person skilled in the art. Preferably, in the bactericidal agent according to the invention, the protein transduction domain is selected from the group consisting of SEQ ID NO: 12 – 25, or a variant thereof, wherein the variant is a functional protein transduction domain and has at least 40%, 45%, 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity with a sequence selected from the group consisting of SEQ ID NO: 12 – 25, respectively.

Preferably, in the bactericidal agent according to the invention, the functional enzymatic domain from a cell wall lytic enzyme is selected from the group consisting of SEQ ID NO: 1 – 7, or a variant thereof, wherein the variant is a functional enzymatic domain from a cell wall lytic enzyme and has at least 40%, 45%, 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity with a sequence selected from the group consisting of SEQ ID NO: 1 – 7, respectively.

Preferably, in the bactericidal agent according to the invention, the cell wall binding domain is selected from the group consisting of SEQ ID NO: 8 – 11, or a variant thereof,

wherein the variant is a functional cell wall binding domain and has at least 40%, 45%, 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity with a sequence selected from the group consisting of SEQ ID NO: 8 – 11, respectively.

Preferably, in the bactericidal agent according to the invention, the antimicrobial peptide is selected from the group consisting of SEQ ID NO: 70 - 90, or a variant thereof, wherein the variant is a functional cell wall binding domain and has at least 40%, 45%, 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity with a sequence selected from the group consisting of SEQ ID NO: 70 - 90, respectively.

A preferred bactericidal agent according to the invention is a bactericidal agent with at least 40%, 45%, 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity with a sequence selected from the group consisting of SEQ ID NO: 27, 28, 29, 30 – 47, or is a bactericidal agent encoded by polynucleotide sequence with at least 40%, 45%, 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity with a sequence selected from the group consisting of SEQ ID NO: 50 – 67.

A preferred bactericidal agent according to the invention is an expression product from a vector with a sequence of at least 40%, 45%, 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity with a sequence selected from the group consisting of SEQ ID NO: 91 - 108.

A further preferred bactericidal agent according to the invention is one selected from the group consisting of a bactericidal agent comprising a protein transduction domain selected from the group consisting of SEQ ID NO: 12 – 25, or a variant thereof, a functional enzymatic domain from a cell wall lytic enzyme selected from the group consisting of SEQ ID NO: 1 – 7, or a variant thereof and, optionally, a cell wall binding domain selected from the group consisting of SEQ ID NO: 8 – 11, or a variant thereof and/or an antimicrobial peptide selected from the group consisting of SEQ ID NO: 70 - 90, or a variant thereof; wherein a variant has at least 40%, 45%, 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity with the respective original sequence.

Further to a method of treatment, this aspect relates to the embodiments of this aspect for the manufacture of a medicament for the treatment of a bacterial infection in a subject in need thereof. Further to a method of treatment, this aspect relates to the embodiments of this aspect for use in the treatment of a bacterial infection in a subject in need thereof.

In a second aspect, the invention provides for a chimeric bactericidal polypeptide comprising a functional enzymatic domain from a cell wall lytic enzyme selected from the group consisting of SEQ ID NO: 1 – 7, or a variant thereof, wherein the variant is a functional enzymatic domain from a cell wall lytic enzyme and has at least 40%, 45%, 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity with a sequence selected from the group consisting of SEQ ID NO: 1 – 7, respectively; and a protein transduction domain selected from the group consisting of SEQ ID NO: 12 – 25, or a variant thereof, wherein the variant is a functional protein transduction domain and has at least 40%, 45%, 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% or 100% sequence identity with a sequence selected from the group consisting of SEQ ID NO: 12 – 25, respectively; and optionally comprising a cell wall binding domain selected from the group consisting of SEQ ID NO: 8 – 11, or a variant thereof, wherein the variant is a functional cell wall binding domain and has at least 40%, 45%, 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity with a sequence selected from the group consisting of SEQ ID NO: 8 – 11, respectively; and/or an antimicrobial peptide selected from the group consisting of SEQ ID NO: 70 - 90, or a variant thereof, wherein the variant is a functional cell wall binding domain and has at least 40%, 45%, 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity with a sequence selected from the group consisting of SEQ ID NO: 70 - 90, respectively.

The invention further provides for a chimeric bactericidal polypeptide having at least 40%, 45%, 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% or 100% sequence identity with a sequence selected from the group consisting of SEQ ID NO: 27, 28, 29, 30 – 47, or a chimeric bactericidal agent encoded by polynucleotide sequence with at least 40%, 45%, 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity with a sequence selected from the group consisting of SEQ ID NO: 50 – 67.

The invention further provides for a chimeric bactericidal polypeptide selected from the group consisting of a bactericidal agent comprising a protein transduction domain selected from the group consisting of SEQ ID NO: 12 – 25, or a variant thereof, a functional enzymatic domain from a cell wall lytic enzyme selected from the group consisting of SEQ ID NO: 1 – 7, or a variant thereof and, optionally, a cell wall binding domain selected from the group consisting of SEQ ID NO: 8 – 11, or a variant thereof and/or an antimicrobial peptide selected from the group consisting of SEQ ID NO: 70 - 90, or a variant thereof; wherein a variant has at least 40%, 45%, 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity with the respective original sequence.

The invention further provides for a polynucleotide encoding a chimeric bactericidal polypeptide according to this aspect of the invention.

The invention further provides for a polynucleotide construct comprising a polynucleotide encoding a chimeric bactericidal polypeptide according to the invention.

The invention further provides for a vector for expression and production of a chimeric bactericidal polypeptide according to the invention. A vector according to the invention preferably comprises a polynucleotide construct according to the invention comprising a polynucleotide encoding a chimeric bactericidal polypeptide according to the invention. A preferred vector according to the invention is a vector with a sequence of at least 40%, 45%, 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity with a sequence selected from the group consisting of SEQ ID NO: 91 - 108.

The invention further provides for a host cell for the production of a chimeric bactericidal polypeptide according to the invention, comprising a polynucleotide construct or a vector according to the invention. The host cell may be any host cell suitable for the production of a chimeric bactericidal polypeptide according to the invention such a prokaryotic and a eukaryotic host cell.

The invention further provides for the production of a chimeric bactericidal polypeptide according to the invention, comprising culturing a host cell according to the invention, comprising a polynucleotide construct or a vector according to the invention under

conditions conducive to the production of the a chimeric bactericidal polypeptide according to the invention and, optionally, isolating and/or purifying the chimeric bactericidal polypeptide produced.

Any suitable route of administration can be used to administer the alkalizing agent according to the invention and the bactericidal agent according to the invention including but not limited to: oral, aerosol or other device for delivery to the lungs, nasal spray, intravenous, intramuscular, intraperitoneal, intrathecal, vaginal, rectal, topical, lumbar puncture, intrathecal, and direct application to the brain and/or meninges. The alkalizing agent according to the invention and the bactericidal agent the invention may be administered to a subject in need thereof or to a cell, tissue or organ of said subject at least once a day, once a week, once a month, once every six months, once a year or whatever regime is suitable for the treatment.

In this document and in its claims, the verb "to comprise" and its conjugations is used in its non-limiting sense to mean that items following the word are included, but items not specifically mentioned are not excluded. In addition, reference to an element by the indefinite article "a" or "an" does not exclude the possibility that more than one of the element is present, unless the context clearly requires that there be one and only one of the elements. The indefinite article "a" or "an" thus usually means "at least one".

The word “about” or “approximately” when used in association with a numerical value (e.g. about 10) preferably means that the value may be the given value (of 10) more or less 0.1% of the value.

All patent and literature references cited in the present specification are hereby incorporated by reference in their entirety.

Herein, sequence identity with a particular sequence preferably means sequence identity over the entire length of said particular polypeptide or polynucleotide sequence. The sequence information as provided herein should not be so narrowly construed as to require inclusion of erroneously identified bases. The skilled person is capable of identifying such erroneously identified bases and knows how to correct for such errors.

"Similarity" between two amino acid sequences is determined by comparing the amino acid sequence and its conserved amino acid substitutes of one peptide or polypeptide to the sequence of a second peptide or polypeptide. Preferably, identity or similarity is calculated over the whole SEQ ID NO as identified herein. "Identity" and "similarity"

can be readily calculated by known methods, including but not limited to those described in Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part I, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heine, G., Academic Press, 1987; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; and Carillo, H., and Lipman, D., SIAM J. Applied Math., 48:1073 (1988).

Preferred methods to determine identity are designed to give the largest match between the sequences tested. Methods to determine identity and similarity are codified in publicly available computer programs. Preferred computer program methods to determine identity and similarity between two sequences include e.g. the GCG program package (Devereux, J., et al., Nucleic Acids Research 12 (1): 387 (1984)), BestFit, BLASTP, BLASTN, and FASTA (Altschul, S. F. et al., J. Mol. Biol. 215:403-410 (1990). The BLAST X program is publicly available from NCBI and other sources (BLAST Manual, Altschul, S., et al., NCBI NLM NIH Bethesda, MD 20894; Altschul, S., et al., J. Mol. Biol. 215:403-410 (1990). The well-known Smith Waterman algorithm may also be used to determine identity.

Preferred parameters for polypeptide sequence comparison include the following: Algorithm: Needleman and Wunsch, J. Mol. Biol. 48:443-453 (1970); Comparison matrix: BLOSSUM62 from Hentikoff and Hentikoff, Proc. Natl. Acad. Sci. USA. 89:10915-10919 (1992); Gap Penalty: 12; and Gap Length Penalty: 4. A program useful with these parameters is publicly available as the "Ogap" program from Genetics Computer Group, located in Madison, WI. The aforementioned parameters are the default parameters for amino acid comparisons (along with no penalty for end gaps).

Preferred parameters for nucleic acid comparison include the following: Algorithm: Needleman and Wunsch, J. Mol. Biol. 48:443-453 (1970); Comparison matrix: matches=+10, mismatch=0; Gap Penalty: 50; Gap Length Penalty: 3. Available as the Gap program from Genetics Computer Group, located in Madison, Wis. Given above are the default parameters for nucleic acid comparisons.

Optionally, in determining the degree of amino acid similarity, the skilled person may also take into account so-called "conservative" amino acid substitutions, as will be clear to the skilled person. Conservative amino acid substitutions refer to the

interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulphur-containing side chains is cysteine and methionine. Preferred conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine. Substitutional variants of the amino acid sequence disclosed herein are those in which at least one residue in the disclosed sequences has been removed and a different residue inserted in its place. Preferably, the amino acid change is conservative. Preferred conservative substitutions for each of the naturally occurring amino acids are as follows: Ala to ser; Arg to lys; Asn to gln or his; Asp to glu; Cys to ser or ala; Gln to asn; Glu to asp; Gly to pro; His to asn or gln; Ile to leu or val; Leu to ile or val; Lys to arg; gln or glu; Met to leu or ile; Phe to met, leu or tyr; Ser to thr; Thr to ser; Trp to tyr; Tyr to trp or phe; and, Val to ile or leu.

A polynucleotide is represented by a nucleotide sequence. A polypeptide is represented by an amino acid sequence.

Table 1. Sequences

SEQ ID NO	Gene / Polypeptide	Sequence
1	EAD Ami2638	MLKHIYSNHIKGNKITAPKPSIQGVVIHNDYGSMTPSQYLPWLYARE NNGTHVNGWASVYANRNEVLWYHPTDYVEWHCGNQWANANLIGFEVC ESYPGRISDKLFLENEEATLKVAADVMKSYGLPVNRNTVRLHNEFFG TSCPHRSWDLHVGKGEPYTTTNINKMKDYFIKRIKHYYD
2	EAD M23-LST	AATHEHSAQWLNNYKKGYGYGPYPLGINGGMHYGVDFFMNIGTPVKA ISSGKIVEAGWSNYGGGNQIGLIENDGVHRQWYMHLSKYNVKVGDYV KAGQIIGWSGSTGYSTAPHLHFQRMVNSFSNSTAQDPMPFLKSAGYG
3	EAD CHAP11	MSIIMEVATMQAKLTKNEFIEWLKTSEGKQFNVDLWYGFQCFDYANA GWKVLFGLLLKGLGAKDIPFANNFDGLATVYQNTPDFLAQPGDMVVF GSNYGAGYGHVAWVIEATLDYIIVYEQNWLGGGWTDGIEQPGWGWEK VTRRQHAYDFPMWFIRPNFK
4	EAD CHAPTw	MKTLKQAESYIKSKVNTGTDFDGLYGYQCMDLAVDYIYHVTDGKIRM WGNAKDAINNSFGGTATVYKNYPAFRPKYGDVVVWTTGNFATYGHIA

		IVTNPDPYGDLQYVTVLEQNWNGNGIYKTELATIRTHDYTGITHFIR PNFA
5	EAD C118II	MGLPSPKKRKPTASEVAAWAKRMIGRRVDVDGYHGAQCWDLPNYIFN RYWHFKTTGNAIAMAWYRYPKGFKFYRNTRNFVPKPGDMAVWGKGSF NNGVGHTAVVIGPSTKSYFTSVDQNWIGANSYTGSPGAKIKHSYNGI SGFVRPPYHA
6	EAD TC187	MALPKTGKPTAKQVVDWAINLIGSGVDVDGYYGRQCWDLPNYIFNRY WNFKTPGNARDMAWYRYPEGFKVFRNTSDFVPKPGDIAVWTGGNYNW NTWGHTGIVVGPSTKSYFYSVDQNWNNSNSYVGSPAAKIKHSYFGVT HFVRPA
7	EAD CHAPK/GH15	MAKTQAEINKRLDAYAKGTVDSPYRIKKATSYDPSFGVMEAGAIDAD GYYHAQCQDLITDYVLWLTDNKVRTWGNAKDQIKQSYGTGFKIHENK PSTVPKKGWIAVFTSGSYQQWGHIGIVYDGGNTSTFTILEQNWNGYA NKKPTKRVDNYYGLTHFIEIPVKA
8	CBD CBD2638	GGKLEVSKAATIKQSDVKQEVKKQEAKQIVKATDWKQNKDGIWYKAE HASFTVTAPEGIITRYKGPWTGHPQAGVLQKGQTIKYDEVQKFDGHV WVSWETFEGETVYMPVRTWDAKTGKVGKLWGEIK
9	CBD CBD118II	APKSKPSKIKTTWNWGGKFTANSTIKVRKSPGLKGIVVESGSWLYKG NYVPFDQVIKKDGYWWIRFKYVQPGSSNKHF
10	CBD CWT-LST	WKTNKYGTLYKSESASFTPNTDIITRTTGPFRSMPQSGVLKAGQTIH YDEVMKQDGHVWVGYTGNSGQRIYLPVRTWNKSTNTLGVLWGTIK
11	CBD CWT-Ale-1	YKTNKYGTLYKSESASFTANTDIITRLTGPFRSMPQSGVLRKGLTIK YDEVMKQDGHVWVGYNTNSGKRVYLPVRTWNESTGELGPLWGTIK
12	CPP 1: Kala Syn	WEAKLAKALAKALAKHLAKALAKALKACEA
13	CPP 2: M918	MVTVLFRRLRIRRASGPPRVRV
14	CPP 3: MAP	KLALKLALKALKAALKLA
15	CPP 4: MPG	GALFLGFLGAAGSTMGAWSQPKKKRKV
16	CPP 5: Penetratin	RQIKIWFQNRRMKWKK
17	CPP 6: Pep-1	KETWWETWWTEWSQPKKKRKV
18	CPP 7: PTD5-Syn	RRQRRTSKLMKR
19	CPP 8: pVEC	LLIILRRRIRKQAHAHSK
20	CPP 9: R_6W_3	RRWWRRWRR
21	CPP10: Polyarginines	(R)n: 6 < n < 15
22	CPP 11: TAT	GRKKRRQRRRPPQ

23	CPP 12: TAT	RKKRRQRRR
24	CPP 13: Transportan	GWTLNSAGYLLGKINLKALAALAKKIL
25	CPP 14: Transportan 10	AGYLLGKINLKALAALAKKIL
26	-	
27	M23-CBD	MAATHEHSAQWLNNYKKGYGYGPYPLGINGGMHYGVDFFMNIGTPVK AISSGKIVEAGWSNYGGGNQIGLIENDGVHRQWYMHLSKYNVKVGDY VKAGQIIGWSGSTGYSTAPHLHFQRMVNSFSNSTAQDPMPFLKSAGY GGKLEVSKAATIKQSDVKQEVKKQEAKQIVKATDWKQNKDGIWYKAE HASFTVTAPEGIITRYKGPWTGHPQAGVLQKGQTIKYDEVQKFDGHV WVSWETFEGETVYMPVRTWDAKTGKVGKLWGEIK
28	CHAP-CBD	MSIIMEVATMQAKLTKNEFIEWLKTSEGKQFNVDLWYGFQCFDYANA GWKVLFGLLLKGLGAKDIPFANNFDGLATVYQNTPDFLAQPGDMVVF GSNYGAGYGHVAWVIEATLDYIIVYEQNWLGGGWTDGIEQPGWGWEK VTRRQHAYDFPMWFIRPNFKGGKLEVSKAATIKQSDVKQEVKKQEAK QIVKATDWKQNKDGIWYKAEHASFTVTAPEGIITRYKGPWTGHPQAG VLQKGQTIKYDEVQKFDGHVWVSWETFEGETVYMPVRTWDAKTGKVG KLWGEIK
29	Ami-CBD	MLKHIYSNHIKGNKITAPKPSIQGVVIHNDYGSMTPSQYLPWLYARE NNGTHVNGWASVYANRNEVLWYHPTDYVEWHCGNQWANANLIGFEVC ESYPGRISDKLFLENEEATLKVAADVMKSYGLPVNRNTVRLHNEFFG TSCPHRSWDLHVGKGEPYTTTNINKMKDYFIKRIKHYYDGGKLEVSK AATIKQSDVKQEVKKQEAKQIVKATDWKQNKDGIWYKAEHASFTVTA PEGIITRYKGPWTGHPQAGVLQKGQTIKYDEVQKFDGHVWVSWETFE GETVYMPVRTWDAKTGKVGKLWGEIK
30	Ami-CBD-Penetratin	MLKHIYSNHIKGNKITAPKPSIQGVVIHNDYGSMTPSQYLPWLYARE NNGTHVNGWASVYANRNEVLWYHPTDYVEWHCGNQWANANLIGFEVC ESYPGRISDKLFLENEEATLKVAADVMKSYGLPVNRNTVRLHNEFFG TSCPHRSWDLHVGKGEPYTTTNINKMKDYFIKRIKHYYDGGKLEVSK AATIKQSDVKQEVKKQEAKQIVKATDWKQNKDGIWYKAEHASFTVTA PEGIITRYKGPWTGHPQAGVLQKGQTIKYDEVQKFDGHVWVSWETFE GETVYMPVRTWDAKTGKVGKLWGEIKELRQIKIWFQNRRMKWKK
31	Ami-CBD-R9	MLKHIYSNHIKGNKITAPKPSIQGVVIHNDYGSMTPSQYLPWLYARE NNGTHVNGWASVYANRNEVLWYHPTDYVEWHCGNQWANANLIGFEVC ESYPGRISDKLFLENEEATLKVAADVMKSYGLPVNRNTVRLHNEFFG TSCPHRSWDLHVGKGEPYTTTNINKMKDYFIKRIKHYYDGGKLEVSK AATIKQSDVKQEVKKQEAKQIVKATDWKQNKDGIWYKAEHASFTVTA

		PEGIITRYKGPWTGHPQAGVLQKGQTIKYDEVQKFDGHVWVSWETFE GETVYMPVRTWDAKTGKVGKLWGEIKELRRRRRRRRR
32	Ami-CBD-TAT	MLKHIYSNHIKGNKITAPKPSIQGVVIHNDYGSMTPSQYLPWLYARE NNGTHVNGWASVYANRNEVLWYHPTDYVEWHCGNQWANANLIGFEVC ESYPGRISDKLFLENEEATLKVAADVMKSYGLPVNRNTVRLHNEFFG TSCPHRSWDLHVGKGEPYTTTNINKMKDYFIKRIKHYYDGGKLEVSK AATIKQSDVKQEVKKQEAKQIVKATDWKQNKDGIWYKAEHASFTVTA PEGIITRYKGPWTGHPQAGVLQKGQTIKYDEVQKFDGHVWVSWETFE GETVYMPVRTWDAKTGKVGKLWGEIKELGRKKRRQRRRPPQ
33	Ami-Penetratin	MLKHIYSNHIKGNKITAPKPSIQGVVIHNDYGSMTPSQYLPWLYARE NNGTHVNGWASVYANRNEVLWYHPTDYVEWHCGNQWANANLIGFEVC ESYPGRISDKLFLENEEATLKVAADVMKSYGLPVNRNTVRLHNEFFG TSCPHRSWDLHVGKGEPYTTTNINKMKDYFIKRIKHYYDGELRQIKI WFQNRRMKWKK
34	Ami-R9	MLKHIYSNHIKGNKITAPKPSIQGVVIHNDYGSMTPSQYLPWLYARE NNGTHVNGWASVYANRNEVLWYHPTDYVEWHCGNQWANANLIGFEVC ESYPGRISDKLFLENEEATLKVAADVMKSYGLPVNRNTVRLHNEFFG TSCPHRSWDLHVGKGEPYTTTNINKMKDYFIKRIKHYYDGELRRRRR RRRR
35	Ami-TAT	MLKHIYSNHIKGNKITAPKPSIQGVVIHNDYGSMTPSQYLPWLYARE NNGTHVNGWASVYANRNEVLWYHPTDYVEWHCGNQWANANLIGFEVC ESYPGRISDKLFLENEEATLKVAADVMKSYGLPVNRNTVRLHNEFFG TSCPHRSWDLHVGKGEPYTTTNINKMKDYFIKRIKHYYDGELGRKKR RQRRRPPQ
36	CHAP11-CBD-Penetratin	MSIIMEVATMQAKLTKNEFIEWLKTSEGKQFNVDLWYGFQCFDYANA GWKVLFGLLLKGLGAKDIPFANNFDGLATVYQNTPDFLAQPGDMVVF GSNYGAGYGHVAWVIEATLDYIIVYEQNWLGGGWTDGIEQPGWGWEK VTRRQHAYDFPMWFIRPNFKGGKLEVSKAATIKQSDVKQEVKKQEAK QIVKATDWKQNKDGIWYKAEHASFTVTAPEGIITRYKGPWTGHPQAG VLQKGQTIKYDEVQKFDGHVWVSWETFEGETVYMPVRTWDAKTGKVG KLWGEIKELRQIKIWFQNRRMKWKK
37	CHAP11-CBD-R9	MSIIMEVATMQAKLTKNEFIEWLKTSEGKQFNVDLWYGFQCFDYANA GWKVLFGLLLKGLGAKDIPFANNFDGLATVYQNTPDFLAQPGDMVVF GSNYGAGYGHVAWVIEATLDYIIVYEQNWLGGGWTDGIEQPGWGWEK VTRRQHAYDFPMWFIRPNFKGGKLEVSKAATIKQSDVKQEVKKQEAK QIVKATDWKQNKDGIWYKAEHASFTVTAPEGIITRYKGPWTGHPQAG VLQKGQTIKYDEVQKFDGHVWVSWETFEGETVYMPVRTWDAKTGKVG KLWGEIKELRRRRRRRRR
38	CHAP11-CBD-TAT	MSIIMEVATMQAKLTKNEFIEWLKTSEGKQFNVDLWYGFQCFDYANA GWKVLFGLLLKGLGAKDIPFANNFDGLATVYQNTPDFLAQPGDMVVF

		GSNYGAGYGHVAWVIEATLDYIIVYEQNWLGGGWTDGIEQPGWGWEK VTRRQHAYDFPMWFIRPNFKGGKLEVSKAATIKQSDVKQEVKKQEAK QIVKATDWKQNKDGIWYKAEHASFTVTAPEGIITRYKGPWTGHPQAG VLQKGQTIKYDEVQKFDGHVWVSWETFEGETVYMPVRTWDAKTGKVG KLWGEIKELGRKKRRQRRRPPQ
39	CHAP11-Penetratin	MSIIMEVATMQAKLTKNEFIEWLKTSEGKQFNVDLWYGFQCFDYANA GWKVLFGLLLKGLGAKDIPFANNFDGLATVYQNTPDFLAQPGDMVVF GSNYGAGYGHVAWVIEATLDYIIVYEQNWLGGGWTDGIEQPGWGWEK VTRRQHAYDFPMWFIRPNFKELRQIKIWFQNRRMKWKK
40	CHAP11-R9	MSIIMEVATMQAKLTKNEFIEWLKTSEGKQFNVDLWYGFQCFDYANA GWKVLFGLLLKGLGAKDIPFANNFDGLATVYQNTPDFLAQPGDMVVF GSNYGAGYGHVAWVIEATLDYIIVYEQNWLGGGWTDGIEQPGWGWEK VTRRQHAYDFPMWFIRPNFKELRRRRRRRRR
41	CHAP11-TAT	MSIIMEVATMQAKLTKNEFIEWLKTSEGKQFNVDLWYGFQCFDYANA GWKVLFGLLLKGLGAKDIPFANNFDGLATVYQNTPDFLAQPGDMVVF GSNYGAGYGHVAWVIEATLDYIIVYEQNWLGGGWTDGIEQPGWGWEK VTRRQHAYDFPMWFIRPNFKELGRKKRRQRRRPPQ
42	M23-CBD-Penetratin	MAATHEHSAQWLNNYKKGYGYGPYPLGINGGMHYGVDFFMNIGTPVK AISSGKIVEAGWSNYGGGNQIGLIENDGVHRQWYMHLSKYNVKVGDY VKAGQIIGWSGSTGYSTAPHLHFQRMVNSFSNSTAQDPMPFLKSAGY GGKLEVSKAATIKQSDVKQEVKKQEAKQIVKATDWKQNKDGIWYKAE HASFTVTAPEGIITRYKGPWTGHPQAGVLQKGQTIKYDEVQKFDGHV WVSWETFEGETVYMPVRTWDAKTGKVGKLWGEIKELRQIKIWFQNRR MKWKK
43	M23-CBD-R9	MAATHEHSAQWLNNYKKGYGYGPYPLGINGGMHYGVDFFMNIGTPVK AISSGKIVEAGWSNYGGGNQIGLIENDGVHRQWYMHLSKYNVKVGDY VKAGQIIGWSGSTGYSTAPHLHFQRMVNSFSNSTAQDPMPFLKSAGY GGKLEVSKAATIKQSDVKQEVKKQEAKQIVKATDWKQNKDGIWYKAE HASFTVTAPEGIITRYKGPWTGHPQAGVLQKGQTIKYDEVQKFDGHV WVSWETFEGETVYMPVRTWDAKTGKVGKLWGEIKELRRRRRRRRR
44	M23-CBD-TAT	MAATHEHSAQWLNNYKKGYGYGPYPLGINGGMHYGVDFFMNIGTPVK AISSGKIVEAGWSNYGGGNQIGLIENDGVHRQWYMHLSKYNVKVGDY VKAGQIIGWSGSTGYSTAPHLHFQRMVNSFSNSTAQDPMPFLKSAGY GGKLEVSKAATIKQSDVKQEVKKQEAKQIVKATDWKQNKDGIWYKAE HASFTVTAPEGIITRYKGPWTGHPQAGVLQKGQTIKYDEVQKFDGHV WVSWETFEGETVYMPVRTWDAKTGKVGKLWGEIKELGRKKRRQRRRP PQ
45	M23-Penetratin	MAATHEHSAQWLNNYKKGYGYGPYPLGINGGMHYGVDFFMNIGTPVK AISSGKIVEAGWSNYGGGNQIGLIENDGVHRQWYMHLSKYNVKVGDY

		VKAGQIIGWSGSTGYSTAPHLHFQRMVNSFSNSTAQDPMPFLKSAGY GELRQIKIWFQNRRMKWKK
46	M23-R9	MAATHEHSAQWLNNYKKGYGYGPYPLGINGGMHYGVDFFMNIGTPVK AISSGKIVEAGWSNYGGGNQIGLIENDGVHRQWYMHLSKYNVKVGDY VKAGQIIGWSGSTGYSTAPHLHFQRMVNSFSNSTAQDPMPFLKSAGY GELRRRRRRRR
47	M23-TAT	MAATHEHSAQWLNNYKKGYGYGPYPLGINGGMHYGVDFFMNIGTPVK AISSGKIVEAGWSNYGGGNQIGLIENDGVHRQWYMHLSKYNVKVGDY VKAGQIIGWSGSTGYSTAPHLHFQRMVNSFSNSTAQDPMPFLKSAGY GELGRKKRRQRRRPPQ
48	-	
49	-	
50	Ami-CBD-Penetratin	Polynucleotide sequence; see sequence listing
51	Ami-CBD-R9	Polynucleotide sequence; see sequence listing
52	Ami-CBD-TAT	Polynucleotide sequence; see sequence listing
53	Ami-Penetratin	Polynucleotide sequence; see sequence listing
54	Ami-R9	Polynucleotide sequence; see sequence listing
55	Ami-TAT	Polynucleotide sequence; see sequence listing
56	CHAP11-CBD-Penetratin	Polynucleotide sequence; see sequence listing
57	CHAP11-CBD-R9	Polynucleotide sequence; see sequence listing
58	CHAP11-CBD-TAT	Polynucleotide sequence; see sequence listing
59	CHAP11-Penetratin	Polynucleotide sequence; see sequence listing
60	CHAP11-R9	Polynucleotide sequence; see sequence listing
61	CHAP11-TAT	Polynucleotide sequence; see sequence listing
62	M23-CBD-Penetratin	Polynucleotide sequence; see sequence listing
63	M23-CBD-R9	Polynucleotide sequence; see sequence listing
64	M23-CBD-TAT	Polynucleotide sequence; see sequence listing
65	M23-Penetratin	Polynucleotide sequence; see sequence listing
66	M23-R9	Polynucleotide sequence; see sequence listing
67	M23-TAT	Polynucleotide sequence; see sequence listing
68	-	
69	-	

70	LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES
71	SMAP-29	RGLRRLGRKIAHGVKKYGPTVLRIIRIAG
72	Indolicidin	ILPWKWPWWPWRR
73	Protegrin	RGGRLCYCRRRFCVCVGR
74	Cecropin P1	SWLSKTAKKLENSAKKRISEGIAIAIQGGPR
75	Magainin	GIGKFLHSAKKFGKAFVGEIMNS
76	Pleurocidin	GWGSFFKKAAHVGKHVGKAALTHYL
77	Cecropin A *A. aegyptae*	GGLKKLGKKLEGAGKRVFNAAEKALPVVAGAKALRK
78	Cecroptin A *D. melanogaster*	GWLKKIGKKIERVGQHTRDATIQGLGIPQQAANVAATARG
79	Buforin II	TRSSRAGLQFPVGRVHRLLRK
80	Sarcotoxin IA	GWLKKIGKKIERVGQHTRDATIQGLGIAQQAANVAATAR
81	Ascaphine	GIKDWIKGAAKKLIKTVASHIANQ
82	Apidaecine	ANRPVYIPPPRPPHPRL
83	Nigrocine	GLLSKVLGVGKKVLCGVSGLVC
84	Pseudin 1	GLNTLKKVFQGLHEAIKLINNHVQ
85	Parasin 1	KGRGKQGGKVRAKAKTRSS
86	Lycotoxin	IWLTALKFLGKHAAKKLAKQQLSKL
87	Ranalexin	FLGGLIVPAMICAVTKKC
88	Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ
89	OR-7	KTYYGTNGVHCTKNSLWGKVRLKNMKYDQNTTYMGRLQDILLGWATG AFGKTFH
90	Buforin I	AGRGKQGGKVRAKAKTRSSRAGLQFPVGRVHRLLRKGNY
91	Ami-CBD-Penetratin	Vector construct: see sequence listing
92	Ami-CBD-R9	Vector construct: see sequence listing
93	Ami-CBD-TAT	Vector construct: see sequence listing
94	Ami-Penetratin	Vector construct: see sequence listing
95	Ami-R9	Vector construct: see sequence listing
96	Ami-TAT	Vector construct: see sequence listing
97	CHAP11-CBD-Penetratin	Vector construct: see sequence listing
98	CHAP11-CBD-R9	Vector construct: see sequence listing

99	CHAP11-CBD-TAT	Vector construct: see sequence listing
100	CHAP11-Penetratin	Vector construct: see sequence listing
101	CHAP11-R9	Vector construct: see sequence listing
102	CHAP11-TAT	Vector construct: see sequence listing
103	M23-CBD-Penetratin	Vector construct: see sequence listing
104	M23-CBD-R9	Vector construct: see sequence listing
105	M23-CBD-TAT	Vector construct: see sequence listing
106	M23-Penetratin	Vector construct: see sequence listing
107	M23-R9	Vector construct: see sequence listing
108	M23-TAT	Vector construct: see sequence listing

FIGURE LEGENDS

Figure 1.

Induction of *S. aureus* SCVs and non-replicating persisters by low pH and bacterial regrowth through pH increase.

MRSA *S. aureus* strains 6850 (a), JE2 (b) and Cowan (c) were inoculated in media buffered at different pH as indicated. Colony phenotypes of viable bacteria were determined and the percentage of SCVs plotted over time. Three independent experiments done in triplicates are presented as mean ± SEM.

Low pH-induced MRSA *S. aureus* strains 6850 (d), JE2 (e) and Cowan (f) persisters were re-inoculated in various buffered pH media as indicated and growth was followed over time. Three independent experiments done in triplicates presented as mean ± SEM.

Figure 2

Intracellular persistence of *S. aureus* within phagolysosomes.

A549 cells were infected with *S. aureus* Cowan and extracellular bacteria were killed by addition of flucloxacillin. The number (a) and phenotype (b) of viable intracellular persisting bacteria were determined at indicated time points. Data are pooled from two experiments performed in triplicates, mean ± SEM.

Figure 3

Reduction of *S. aureus* persisters through phagolysosome alkalinization. *S. aureus* Cowan-infected A549 cells were treated with flucloxacillin alone (control) or supplemented with lysosomotropic alkalinizing agents (chloroquine (a), bafilomycin A1 (b) and ammonium chloride (c)). Colony phenotypes of viable intracellular persisting bacteria were determined and enumerated at indicated time points. Data were pooled from three independent experiments done in triplicates, mean ± SEM. Two-way ANOVA found the factors time and treatment to be significant (p-value < 0.01).

Figure 4

Reduction of *S. aureus* persisters by chloroquine in an *in vivo* infection model.

Mice were infected with *S. aureus* Cowan intraperitoneally. Six hours and two days post-infection mice were treated with 1 mg flucloxacillin and 0.2 mg chloroquine (+ CQ). Mice treated with flucloxacillin only served as control (-CQ). †, sacrifice (a). Colony phenotypes of bacteria recovered from target tissues (b), peripheral blood and peritoneal lavage (c) were determined and enumerated. Each point represents one mouse. Horizontal bars indicate mean ± SEM, n = 11 mice per group. PL, peritoneal lavage. Two-way ANOVA found the factor treatment to be significant (p-value < 0.01).

Figure 5

Intracellular targeting of S. aureus in osteosarcoma cells by mixtures of engineered endolysins. (A) Cells infected for 3h with *S. aureus* Newman (MOI 0.1) treated by endolysin mixtures for 1h and 4h. (B) Cells infected for 3h with *S. aureus* Newman (MOI 0.1) treated by endolysin mixtures for 1h and 4h in the presence of 20 μM chloroquine. (C) Cells infected for 3h with *S. aureus* Cowan (MOI 0.01) treated by endolysin mixtures for 1h and 4h. (D) Cells infected for 3h with *S. aureus* Cowan (MOI 0.01) treated by endolysin mixtures for 1h and 4h in the presence of 20 μM chloroquine.

Figure 6

Intracellular targeting of S. aureus in osteosarcoma cells (MOI 1.0) by mixtures of engineered endolysins. (A) Cells infected for 24h with *S. aureus* Newman treated by endolysin mixtures for 4h. (B) Cells infected for 24h with *S. aureus* Newman treated by

endolysin mixtures for 4h in the presence of 20 μM chloroquine. (C) Cells infected for 72h with *S. aureus* Cowan treated by endolysin mixtures for 4h. (D) Cells infected for 72h with *S. aureus* Cowan treated by endolysin mixtures for 4h in the presence of 20 μM chloroquine.

Figure 7

Activity of bactericidal agents according to the invention comprising a functional enzymatic domain from a cell wall lytic enzyme and further comprising a protein transduction domain on the N-terminal side of the molecule: (A) R9-CHAP-CBD, (B) R9-M23-CBD, (C) TAT-Ami-CBD, (D) TAT-CHAP-CBD, and (E) TAT-M23-CBD.

EXAMPLES

The present invention is further described by the following examples which should not be construed as limiting the scope of the invention.

Unless stated otherwise, the practice of the invention will employ standard conventional methods of molecular biology, virology, microbiology or biochemistry. Such techniques are described in Sambrook *et al.* (1989) Molecular Cloning, A Laboratory Manual (2nd edition), Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press; in Sambrook and Russell (2001) *Molecular Cloning: A Laboratory Manual,* Third Edition, Cold Spring Harbor Laboratory Press, NY; in Volumes 1 and 2 of Ausubel *et al.* (1994) *Current Protocols in Molecular Biology, Current Protocols*, USA; and in Volumes I and II of Brown (1998) *Molecular Biology LabFax*, Second Edition, Academic Press (UK); *Oligonucleotide Synthesis* (N. Gait editor); *Nucleic Acid Hybridization* (Hames and Higgins, eds.).

Example 1.

Induction of *Staphylococcus aureus* persisters by low pH, awakening by phagolysosomal alkalinization and effective treatment by phagolysosomal alkalinization combined with a bactericidal agent.

1.1 pH-dependent induction of *S. aureus* Small Colony Variants (SCVs)

The well-defined MSSA strains 6850 and Cowan and MRSA strain JE2 were grown in 4.0, 5.5, 6.5 and 7.4 pH media, mimicking the pH found in physiologic sites such as lysosomes, abscesses and blood. Directly after inoculation *S. aureus* showed a large colony phenotype, independent of the pH. The frequency of SCVs significantly increased over time in pH 4.0 growth medium and reached 39% (JE2 and 6850) and 28% (Cowan) after five days. In contrast pH 7.4 growth medium sustained SCVs below 2% in all strains tested (Fig. 1a - c). An intermediate percentage of SCVs was found at pH 5.5 and 6.5. Thus, we showed a clear correlation between low pH and SCV formation. This method permitted easy and controlled formation of unstable, non-genetically modified SCVs in various *S. aureus* strains.

1.2 Induction of non-replicating *S. aureus* by low pH

The pH-dependent growth of *S. aureus* Cowan was followed over time by labeling the bacterial cell wall with fluorescent cell wall binding domains (CBDs). Immediately after staining, bacterial cell walls were fully labeled. After three days in pH 4.0 growth medium, the majority of bacteria still exhibited fluorescent cell walls, consistent with absent bacterial replication. In contrast, bacteria grown at pH 7.4 proliferated extensively, demonstrated by highly fragmented and89 reduced fluorescent cell wall labeling . Scanning electron microscopy (SEM) of bacteria originating from a small colony, obtained under low-pH conditions, showed impaired cell division resulting in rod-shaped *S. aureus*, in contrast to large colony bacteria showing normal cell division.

1.3 Growth resumption of low pH-adapted *S. aureus* persisters

Our findings indicated that both, SCVs and non-replicating persisters, are induced by low pH. In clinics, the presence of these persisting bacteria correlates with increased recurrence of infection which implies that bacteria revert to a highly virulent and fast-growing form 14. We therefore tested *in vitro* whether low pH-induced SCVs and/or non-replicating persisters can restore normal growth in neutral pH. Non-replicating *S. aureus* persisters were induced and kept at pH 4.0 for three days and then transferred to pH 4.0, 5.5, 6.5 or 7.4 growth media. Bacteria in pH 7.4 and 6.5 resumed growth after approximately 12 hours (Fig. 1d - f) whereas bacteria kept at low pHs (< 6.5) remained in a nonproliferating state (Fig. 1d - f). These data suggest that both persisting phenotypes

were reversible adaptions to low pH as supported by the capability of regrowth upon neutralization of pH.

1.4 Intracellular induction of *S. aureus* SCVs

We investigated whether internalized *S. aureus* exhibited a SCV phenotype. MSSA strain Cowan is highly invasive, but not cytotoxic which allowed maintaining this strain intracellularly over several days in the lung epithelial cell line A549. Extracellular bacteria were killed by adding a high dose of flucloxacillin to the infected host cells. Flucloxacillin is typically used to treat *S. aureus* endocarditis in patients. Absence of extracellular bacteria was confirmed by sterility of culture supernatants. Host cells were lysed to release intracellular bacteria and colony counts, as well as colony phenotypes, were determined at various time points. Five hours after infection, 0.2% of all viable intracellular bacteria had a SCV phenotype (Fig. 2a - b). The number of viable intracellular persisting bacteria decreased during the course of infection while the frequency of SCVs increased and reached 5.6% after seven days.

1.5 Phagolysosomal localization of persisting *S. aureus*

Our data indicated that acidity favored SCV formation, suggesting that the acidic phagolysosomal milieu may have the same effect. A549 cells were infected with *S. aureus* Cowan. Intracellular bacteria were localized within LAMP-2 antibody positive vesicles, visualized by fluorescence microscopy. LAMP-2 (CD 107b) is highly expressed in phagolysosomes, suggesting that intracellular persisting *S. aureus* predominantly resided within phagolysosomes.

1.6 Reduction of *S. aureus* SCVs through phagolysosomal alkalinization

Since low pH induced SCVs and/or non-replicating *S. aureus* and medium pH neutralization resulted in bacterial regrowth, we treated infected host cells with lysosomotropic alkalinizing agents. Chloroquine, bafilomycin A1 or ammonium chloride all neutralized the phagolysosomal pH. Host cells treated with lysosomotropic alkalinizing agents exhibited significantly lower percentages of SCVs seven days after infection (Fig. 3). No differences in total colony counts between control and treated cells were observed. Lysosomotropic alkalinizing agents did not inhibit bacterial growth at the

concentrations used. No significant differences in host cell viability were observed after treatment with the alkalinizing agents.

1.7 Growth resumption of intra-phagolysosomal persisting *S. aureus* by chloroquine treatment resulting in reduction of SCV percentages in cells and in mice

Growth resumption of *S. aureus* persisters through treatment of host cells with chloroquine was assessed. Fluorescence microscopy revealed that *S. aureus* localized within phagolysosomes in both, control and chloroquine-treated host cells, three days post-infection. We observed no dividing bacteria in infected host cells without chloroquine treatment. However, chloroquine facilitated bacterial cell division as assessed by transmission electron microscopy (TEM).

Mice infected with *S. aureus* Cowan were treated with flucloxacillin alone (control), or in combination with chloroquine (Figure 5a). Chloroquine treatment significantly reduced the frequency of SCVs in mice in various organs (Fig. 5b) and compartments (Fig. 5c). Absolute bacterial numbers were comparable, independent of chloroquine treatment

1.8 Discussion

This study showed that low pH, as found in abscesses and within lysosomes, induced the persisting *S. aureus* subpopulations SCVs and non-replicating persisters. Raising pH in the culture medium or within the phagolysosomes using alkalinizing agents reverted *S. aureus* to normal growth. SCV formation was shown to be triggerd by antibiotic pressure. In addition, extreme environmental stresses such as prolonged exposure to low temperature, very acidic or alkaline environments, or osmotic stress may trigger SCV and/or persister formation in *S. aureus* and coagulase-negative staphylococci. These observations, together with our new findings, show how multiple stimuli lead to *S. aureus* persister formation. Localization within the host cell shields *S. aureus* from commonly used antibiotics such as the extracellularly active beta-lactams with poor cell penetration In addition, the low intraphagolysosomal pH renders antibiotics with intracellular activity such as clindamycin and fluoroquinolones less active. We found that the addition of lysosomotropic alkalinizing agents to the usually prescribed antibiotics such as flucloxacillin reduced the frequency of *S. aureus* SCVs *in vitro* as well as *in vivo*. We thus identified a simple strategy to circumvent the host dependent component of S.aureus

persister formation. In clinical settings, the presence of SCVs in osteomyelitis and device-related infections has been associated with increased relapse rates, despite administration of antibiotics. Bacteria adapt to antibiotic stress by SCV and/or persister formation. We now showed that *S. aureus* SCVs and non-replicating persisters retained the ability to revert to a highly virulent and fast-growing form. The capacity to revert to fast growth (phenotype switching) results in relapsing infection. In addition, it renders identification of SCVs difficult. Further aggravating the SCV problem in clinics is underestimation of SCVs in clinical microbiology laboratories, since they form tiny and thus difficult to detect colonies which are easily overgrown by their fast growing counterparts . We postulate that the addition of alkalinizing agents to the usually prescribed antibiotics will reduce the frequency of SCVs and could therefore reduce recurrence rates in the future.. Persisting bacteria are not unique to *S. aureus* but have also been described to occur in various other human pathogens, such as Salmonella spp., *Pseudomonas aeruginosa*, *Escherichia coli* and *Mycobacterium tuberculosis*. In addition to low pH, bacterial persisters can arise due to mechanisms that include the toxin-antitoxin systems. Accordingly, activation of a SOS response (ppGpp) in response to DNA damage due to oxidative stress results in decreased ATP levels. This leads to the shutdown of metabolism resulting in reduced growth. Various toxin-antitoxin modules are activated by acidification and/or nutrient starvation in Salmonella, causing formation of persisters. In accordance with our findings, Salmonella persister formation has been reported in macrophages triggered by the acidic and nutritionally poor environment of the Salmonella-containing vacuole that was reversible by addition of bafilomycin A1 44. In contrast to bafilomycin A1, chloroquine is routinely used in patients to treat malaria as well as some rheumatic diseases. Phagolysosomal pH neutralization with chloroquine may therefore provide a novel therapeutic eradication strategy against intracellular persisting staphylococcal reservoirs.

Example 2.

Effective treatment of *Staphylococcus aureus* persisters by phagolysosomal alkalinization combined with a bactericidal agent comprising a protein transduction domain.

The bactericidal agents according to the invention with a protein transduction domain for efficient delivery into the infected host cell with a sequence selected from the group

consisting of SEQ ID NO: 30 – 47 are used for combination with phagolysosomal pH neutralization for treatment of intracellular *S. aureus* infection *in vitro* and *in vivo*. The treatment results in effective treatment of the intracellular *S. aureus* infection, with some variety in the efficiency depending on the specific bactericidal agent according to the invention used.

Example 3.

Effective treatment of intracellular infection with *Staphylococcus aureus*.

The bactericidal agents according to the invention with a protein transduction domain for efficient delivery into the infected host cell with a sequence selected from the group consisting of SEQ ID NO: 27 – 47 were used for treatment of intracellular *S. aureus* infection, either combined or not with phagolysosomal pH neutralization.

Method intracellular *S. aureus* killing assay.

S. aureus was grown in LB broth at 37 °C, shaking at 220 rpm, overnight. The overnight culture was diluted in fresh LB (1:10) and grown further for 2 h. Then, the bacteria were centrifuged, the pellet was washed with PBS and the culture was re-suspended in PBS with the OD600 adjusted to 0.4 (c.a. 2×10^8 CFU/mL). Bacterial cells were sonicated prior to infection (SONOPULS HD 2070) for 1 minute with 1 second pulses at 40% of the power. MG-63 osteosarcoma cells were grown in 12-well dishes with the amount of 5×10^5 cells/well in 1 mL EMEM culture media with 10 % fetal bovine serum (FBS) for 24 h prior infection. Then, the cells were infected with *S. aureus* Newman and Cowan at the following conditions: (A) *S. aureus* Newman at MOI of 0.1 for 3 h, (B) *S. aureus* Newman at MOI of 1.0 for 24 h, and (C) *S. aureus* Cowan at MOI of 1.0 for 72 h. The plates were centrifuged at 1200 rpm for 5 min and incubated at 37 °C with the flush of CO2. After invasion, eukaryotic cells were washed 3× with PBS to remove remaining extracellular *S. aureus* and exposed to floxacillin (1 mg/mL) for 2 h to kill any left non-internalized bacteria. For each experiment (A, B, and C) one part of the samples was exposed to chloroquine treatment (20 μM) to increase lysosomal pH and evaluate its effect on further treatment with endolysins. The supernatant from the antibiotic-treated cells was plated to check for floxacillin treatment efficiency. Then, the eukaryotic cells were again washed with PBS (3×) to remove dead bacteria and subjected to endolysin treatment. The composition of applied endolysin preparations is summarized in Table 2.

Eukaryotic cells were treated with 1 mL of 1 μM endolysin preparation (diluted in EMEM supplemented with 1 mg/mL floxacillin and +/- 20 μM chloroquine) for (A) 1h and 4h, (B and C) 4h. The control was treated with 1 mL of 1 mg/mL floxacillin, +/- 20 μM chloroquine in EMEM. The cultures were then washed 3× with PBS and examined under microscope to determine if there had been osteoblast cell lysis. Next, they were trypsinized (Trypsin-EDTA 0.25%, Gibco®) and lysed with 800 μL 0.1 % Triton X-100. The cell lysate was subjected to serial dilution plating on LB and overnight incubation at 37 °C.

Table 2 Composition of the endolysin mixtures used for intracellular eradication of *S. aureus*.

Components of the endolysin mixture	**Ratio**	**Concentration of each component**
CHAP-CBD + M23-CBD	1:1	500nM:500nM
CHAP-CBD-TAT + M23-CBD-TAT	1:1	500nM:500nM
CHAP-CBD-R9 + M23-CBD-R9	1:1	500nM:500nM
CHAP-CBD-Penetratin + M23-CBD-Penetratin	1:1	500nM:500nM
CHAP-TAT + M23-TAT + Ami-TAT	1:1:1	333nM:333nM:333nM
CHAP-R9 + M23-R9 + Ami-R9	1:1:1	333nM:333nM:333nM
CHAP-Penetratin + M23-Penetratin + Ami-Penetratin	1:1:1	333nM:333nM:333nM

Results

Successful expression and purification of all protein constructs was achieved. The summary of all expressed endolysin constructs with the corresponding molecular weights and concentrations is shown in Table 3. Relatively high concentrations were obtained for most of the endolysin constructs, implying that a correct protein expression and purification strategy was used.

Table 3 Summary of all expressed endolysin constructs with the corresponding molecular weight and concentration

SEQ ID NO:	**Protein**	**Mol. Weight (KDa)**	**Concentration (mg/ml)**	**Concentration (μM)**
27	M23-CBD	29.973	3.82	127.4
28	CHAP-CBD	33.088	2.36	71.33

29	Ami-CBD	35.385	3.30	93.26
44	M23-CBD-TAT	31.916	1.13	34.30
38	CHAP-CBD-TAT	35.031	1.85	52.81
32	Ami-CBD-TAT	37.328	0.40	10.71
43	M23-CBD-R9	31.620	0.45	14.23
37	CHAP-CBD-R9	34.736	1.05	30.20
31	Ami-CBD-R9	37.033	0.30	8.10
42	M23-CBD-Penetratin	32.444	0.56	17.26
36	CHAP-CBD-Penetratin	35.559	2.03	57.08
30	Ami-CBD-Penetratin	37.856	0.41	10.82
47	M23-TAT	17.485	1.26	72.00
41	CHAP-TAT	20.600	1.44	69.90
35	Ami-TAT	22.897	0.83	36.25
46	M23-R9	17.189	2.51	146.00
40	CHAP-R9	20.305	0.83	40.88
34	Ami-R9	22.601	0.76	33.63
45	M23-Penetratin	18.012	0.80	44.41
39	CHAP-Penetratin	21.128	0.73	34.55
33	Ami-Penetratin	23.424	0.96	41.00

All expressed endolysins were effective in killing *S. aureus* Cowan in plate assay and in time-killing assay.

Intracellular *S. aureus* killing assay

The mixtures of endolysin constructs as listed in Table 2 were further tested for their bacterial killing efficacy in cell tissue cultures.

In our model MG-63 osteosarcoma cells were used to mimic the condition of osteomyelitis. Cells were first infected for a certain time with the pathogen S. aureus Newman or Cowan and then treated with the endolysin mixture in presence or absence of chloroquine. We expected that increasing intracellular pH by application of chloroquine would create more favorable conditions for endolysin activity.

Osteosarcoma cells were exposed to *S. aureus* Newman and Cowan for 3 h followed by 2 h floxacillin treatment to inactivate any non-internalized bacteria. Then, the tissue cells were treated for 1 h and 4 h with 1 μM of endolysin mixture with and without

chloroquine. The results are shown in Figure 5. Figure 5A shows the result of treatment of *S. aureus* Newman by endolysin mixtures exclusively and Figure 5B shows the result of a combination treatment with chloroquine. Clearly, 4h treatment was more effective in both experimental settings. Moreover, endolysin treatment in the presence of chloroquine resulted in greater decrease in bacterial counts. Interestingly, mixture of the CPP-free endolysins showed a very good killing efficacy, which was not expected. It is, however, possible that the vast amount of positive charge associated with CBD attracts the enzyme to the negatively charged cell membrane and induces its intracellular translocation. Such a putative mechanism for CPP-free endolysin translocation is the key of the translocation of CPPs. All endolysin mixtures displayed similar killing properties in this assay, even though their activity differed substantially depending on the presence of CBD and type of CPP tag. It is likely that different constructs have different penetrating properties, depending on the CPP, EAD and CBD presence. For instance, EAD-CBD mixture has very high activity but might not be transported via the cell membrane as efficient as any CPP-containing variants. Whereas, EAD-CPP constructs do not have as high activity but are smaller and contain the transduction domain, which make their intracellular transport more efficient and their diffusivity better due to the lack of immobilization of the enzyme on the cell surface mediated by CBD binding. As a result, enzymes with different properties might have given similar results eventually.

Figure 5C and Figure 5D show the results of the same experiment performed with a different strain of the pathogen: S. aureus Cowan. In this case, the treatment did not seem to be effective. For both samples, without chloroquine (Figure 5C) and with chloroquine (Figure 5D), the decrease in the viable counts is negligible. Only treatment with the mixture of CHAP-CBD-R9 + M23-CBD-R9 in presence of chloroquine resulted in a significant eradication of the pathogen. It is known that *S. aureus* Cowan resides intracellularly in the lysosomes. It is very likely that intracellular localization of *S. aureus* Newman is different – it may just reside in the cytoplasm, hence the results of the two experiments are different. Moreover, the intracellular fate of CPP-tagged endolysin constructs is unknown at this point, therefore one may only speculate that endolysin constructs fused with the R9 tag are the only ones that accumulate in the lysosomes, hence they showed good killing properties against *S. aureus* Cowan.

To simulate a real-life infection osteosarcoma tissue cells were infected with *S. aureus* for 24 h and 72 h before the treatment. Such a long infection time allowed the bacteria to

settle intracellularly in their final destinations. Again, the cells were treated with the mixtures of endolysin constructs in presence and in absence of chloroquine. Figure 6A and Figure 6B show the results of the treatment of osteosarcoma cells infected for 24 h with *S. aureus* Newman in absence and in presence of chloroquine, respectively. Figure 6C and Figure 6D correspond to the results of treated cells infected for 72 h with *S. aureus* Cowan. In both cases treatment with endolysin mixtures seems effective, especially with the addition of chloroquine. The best result was obtained for the treatment of *S. aureus* Newman with EAD-CBD-R9 mixture in the presence of chloroquine, where ca. 60 % of the pathogen was eradicated (Figure 6B). Equally good results were achieved for *S. aureus* Cowan treated with the mixtures of EAD-CBD, EAD-CBD-TAT, EAD-CBD-R9 and EAD-CBD-Penetratin, where around 50 % of the pathogen was killed. In general, CBD-containing endolysin constructs displayed better killing efficacy, including EAD-CBD mixture, not fused with the transduction domain.

Altogether it was demonstrated that treatment of intracellular *S. aureus* by engineered endolysins together with chloroquine was more effective that without chloroquine. Application of such alkalizing agents likely enhances the activity of lytic enzymes intracellularly and the combinatorial treatment of endolysins with chloroquine could be a good alternative to conventional antibiotic therapy.

In conclusion, this study showed the potential of CPP-fused endolysins in treatment of intracellular and extracellular *S. aureus*. Such a method of therapy could be used to treat patients with conditions like e.g. osteomyelitis, endocarditis, bacteremia or sepsis, as well as bovine mastitis in dairy cattle.

Example 4.

Several bactericidal agents according to the invention comprising a functional enzymatic domain from a cell wall lytic enzyme and further comprising a protein transduction domain on the N-terminal side of the molecule were prepared and analyzed according to methods as described elsewhere herein (R9-CHAP-CBD, R9-M23-CBD, TAT-Ami-CBD, TAT-CHAP-CBD, and TAT-M23-CBD). All data were collected in turbidity reduction assays on *S. aureus* SA113 substrate cells and at 100nM protein concentrations (except Ami-CBD constructs where 1 μM was used) according as described previously in WO2013/169104. Figure 7 depicts the performance of the constructs. As can be observed from Figure 7, in general, the N-terminal CPP tagged proteins are active, but

perform less compared to un-tagged or C-terminal tagged variants when analyzed on *S. aureus* cells. To analyze the efficiency of intracellular killing, the constructs should be tested in an intracellular *S. aureus* killing assay as in example 3.

REFERENCE LIST

1. Dantes, R., et al. National burden of invasive methicillin-resistant Staphylococcus aureus infections, United States, 2011. JAMA internal medicine 173, 1970-1978 (2013).

2. Fowler, V.G., Jr., et al. Persistent bacteremia due to methicillin-resistant Staphylococcus aureus infection is associated with agr dysfunction and low-level in vitro resistance to thrombin-induced platelet microbicidal protein. The Journal of infectious diseases 190, 1140- 1149 (2004).

3. Welsh, K.J., et al. Predictors of relapse of methicillin-resistant Staphylococcus aureus bacteremia after treatment with vancomycin. Journal of clinical microbiology 49, 3669-3672 (2011).

4. Libraty, D.H., Patkar, C. & Torres, B. Staphylococcus aureus reactivation osteomyelitis after 75 years. The New England journal of medicine 366, 481-482 (2012).

5. Proctor, R.A., et al. Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. Nat Rev Microbiol 4, 295-305 (2006).

6. Proctor, R.A., van Langevelde, P., Kristjansson, M., Maslow, J.N. & Arbeit, R.D. Persistent and relapsing infections associated with small-colony variants of Staphylococcus aureus. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 20, 95-102 (1995).

7. Conlon, B.P. Staphylococcus aureus chronic and relapsing infections: Evidence of a role for persister cells: An investigation of persister cells, their formation and their role in S. aureus disease. BioEssays : news and reviews in molecular, cellular and developmental biology 36, 991-996 (2014).

8. Fauvart, M., De Groote, V.N. & Michiels, J. Role of persister cells in chronic infections: clinical relevance and perspectives on anti-persister therapies. Journal of medical microbiology 60, 699-709 (2011).

9. Conlon, B.P., et al. Activated ClpP kills persisters and eradicates a chronic biofilm infection. Nature 503, 365-370 (2013).

10. Lechner, S., Lewis, K. & Bertram, R. Staphylococcus aureus persisters tolerant to bactericidal antibiotics. Journal of molecular microbiology and biotechnology 22, 235-244 (2012).

11. Kaiser, P., et al. Cecum lymph node dendritic cells harbor slow-growing bacteria phenotypically tolerant to antibiotic treatment. PLoS biology 12, e1001793 (2014).

12. Singh, R., Ray, P., Das, A. & Sharma, M. Role of persisters and small-colony variants in antibiotic resistance of planktonic and biofilm-associated Staphylococcus aureus: an in vitro study. Journal of medical microbiology 58, 1067-1073 (2009).

13. Garcia, L.G., et al. Antibiotic activity against small-colony variants of Staphylococcus aureus: review of in vitro, animal and clinical data. The Journal of antimicrobial chemotherapy 68, 1455-1464 (2013).

14. Tuchscherr, L., et al. Staphylococcus aureus phenotype switching: an effective bacterial strategy to escape host immune response and establish a chronic infection. Embo Molecular Medicine 3, 129-141 (2011).

15. Vesga, O., et al. Staphylococcus aureus small colony variants are induced by the endothelial cell intracellular milieu. J Infect Dis 173, 739-742 (1996).

16. Tuchscherr, L., et al. Staphylococcus aureus small-colony variants are adapted phenotypes for intracellular persistence. The Journal of infectious diseases 202, 1031-1040 (2010).

17. Baudoux, P., et al. Combined effect of pH and concentration on the activities of gentamicin

326 and oxacillin against Staphylococcus aureus in pharmacodynamic models of extracellular and intracellular infections. The Journal of antimicrobial chemotherapy 59, 246-253 (2007).

18. Lam, C. & Mathison, G.E. Effect of low intraphagolysosomal pH on antimicrobial activity of antibiotics against ingested staphylococci. Journal of medical microbiology 16, 309-316 (1983).

19. Nguyen, H.A., et al. Factors influencing the intracellular activity of fluoroquinolones: a study using levofloxacin in a Staphylococcus aureus THP-1 monocyte model. The Journal of antimicrobial chemotherapy 57, 883-890 (2006).

20. Barcia-Macay, M., Seral, C., Mingeot-Leclercq, M.P., Tulkens, P.M. & Van Bambeke, F. Pharmacodynamic evaluation of the intracellular activities of antibiotics against Staphylococcus aureus in a model of THP-1 macrophages. Antimicrobial agents and chemotherapy 50, 841-851 (2006).

21. Lebeaux, D., et al. pH-mediated potentiation of aminoglycosides kills bacterial persisters and eradicates in vivo biofilms. The Journal of infectious diseases 210, 1357-1366 (2014).

22. Schroeder, A., Kland, R., Peschel, A., von 340 Eiff, C. & Aepfelbacher, M. Live cell imaging of phagosome maturation in Staphylococcus aureus infected human endothelial cells: small colony variants are able to survive in lysosomes. Medical Microbiology and Immunology 195, 185-194 (2006).

23. Kahl, B.C., et al. Thymidine-dependent Staphylococcus aureus small-colony variants are associated with extensive alterations in regulator and virulence gene expression profiles. Infect Immun 73, 4119-4126 (2005).

24. Parret, A.H. et al. Bacteria killing their own kind: novel bacteriocins of *Pseudomonas* and other γ-proteobacteria. Trends in Microbiology 10, 107-112 (2002)

25. Naz S.A. et al. Biophysicochemical characterization of Pyocin SA189 produced by *Pseudomonas aeruginosa* SA189. Brazilian Journal of Microbiology 46, 1147-1154 (2015).

CLAIMS

1. Use of an effective amount of an alkalizing agent that increases the intracellular pH of a host cell and/or of an intracellular compartment of a host cell and an effective amount of a bactericidal agent in the manufacture of a medicament for the treatment of a bacterial infection in a subject in need thereof, wherein the bactericidal agent is selected from the group consisting of a subclass IIIa bacteriocin, a bacterial autolysin and a bacteriophage lysin, and wherein the agent that increases the intracellular pH and/or the pH of the intracellular compartment is a lysosomotropic alkalizing agent.

2. The use according to claim 1, wherein the bacterial infection is an intracellular and/or persistent bacterial infection and/or is a *Staphylococcus* infection, preferably an *S. aureus* infection.

3. The use according to claim 1 or 2, wherein the host cell is a eukaryotic host cell within the subject in need of treatment and/or wherein the intracellular compartment is a phagolysosome.

4. The use according to any of the preceding claims, wherein the lysosomotropic alkalizing agent is selected from the group of chloroquine, bafilomycin A1, and ammonium chloride.

5. The use according to any of the preceding claims, wherein the bactericidal agent is a bactericidal agent capable of entering the host cell and/or the intracellular compartment of the host cell.

6. The use according to claim 1 to 5, wherein the bactericidal agent comprises a functional enzymatic domain from a cell wall lytic enzyme and preferably further comprises a protein transduction domain.

7. The use according to claim 6, wherein the bactericidal agent further comprises an antimicrobial peptide.

8. The use according to claim 6 or 7, wherein the bactericidal agent further comprises a cell wall binding domain.

9. The use according to claim 6 - 9, wherein the protein transduction domain is selected from the group consisting of SEQ ID NO: 12 - 25.

10. The use according to claim 6 - 9, wherein the functional enzymatic domain from a cell wall lytic enzyme is selected from the group consisting of SEQ ID NO: 1 - 7.

11. The use according to claim 8 - 10, wherein the cell wall binding domain is selected from the group consisting of SEQ ID NO: 8 - 11.

12. The use according to claim 7 - 11, wherein the antimicrobial peptide is selected from the group consisting of SEQ ID NO: 70 - 90.