(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
15 June 2017 (15.06.2017)

(10) International Publication Number
WO 2017/098474 A1

(51) International Patent Classification:
A61K 9/00 (2006.01) *A61K 31/132* (2006.01)
A61K 47/36 (2006.01)

(21) International Application Number:
PCT/IB2016/057508

(22) International Filing Date:
9 December 2016 (09.12.2016)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
109026 9 December 2015 (09.12.2015) PT

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*
— *with sequence listing part of description (Rule 5.2(a))*

WO 2017/098474 A1

(54) Title: ANTIMICROBIAL PEPTIDE-LOADED HYALURONIC ACID-BASED FORMULATIONS, METHOD OF PRODUCTION AND USES THEREOF

(57) Abstract: The present disclosure relates to a novel formulation for the delivery of an antimicrobial peptide (AMP) for the treatment of infections, particularly in the lungs and/or airways. More particularly, it relates to a peptide loaded in hyaluronic acid polymer, preferably antimicrobial peptide encapsulated in hyaluronic acid nanoparticles. This solution may be used in medicine or as a medicament.

DESCRIPTION

ANTIMICROBIAL PEPTIDE-LOADED HYALURONIC ACID-BASED FORMULATIONS, METHOD OF PRODUCTION AND USES THEREOF

Technical domain

[0001] The present solution relates to a novel formulation for the delivery of an antimicrobial peptide (AMP) for the treatment of infections, particularly in the lungs and/or airways. Said peptide is preferentially a cathelicidin analogue. More particularly, it relates to a peptide loaded in hyaluronic acid polymer, preferably antimicrobial peptide encapsulated in hyaluronic acid nanoparticles. Alternatively, said nanoparticles can be loaded with further therapeutic agent. This solution may be used in pharmacy and medicine.

Background

[0002] Infectious diseases represent a major global concern, as they still represent a cause of increased morbidity and mortality worldwide. Moreover, the emergence of antibiotic-resistant bacterial strains urged the need to develop better therapeutic approaches for infection treatment. Tuberculosis (TB), a disease caused by the highly virulent *Mycobacterium tuberculosis*, currently has recently joined HIV/AIDS as the world's deadliest infectious diseases. The most recent data provided by the World Health Organization reports that around 9 million people were diagnosed with TB in 2013, having 1.1 million of those died from the disease. Additionally, as a result of overuse and misuse of antibiotics, multi-drug resistant TB (MDR-TB) strains are emerging at a rate of about 3.5% over recent years.

[0003] Bacille Calmette-Guérin (BCG) is the only vaccine available and although it is quite effective in preventing childhood TB, new infections have been found in adults. Current standard treatments rely on an intensive 2-month administration of a multi-drug cocktail (referred as the first-line therapy) comprising isoniazid, pyrazinamide, rifampicin and ethambutol, followed by a 4-month intake of rifampicin and isoniazid. Treatment for MDR-TB is based on the administration of pyrazinamide together with second-line drugs, such as

ethionamide, prothionamide, cycloserine, capreomycin or fluoroquinolones. In this case, treatment can last up to 24 months. Noteworthy, whereas first-line TB treatment generally costs around $22 per patient, MDR-TB treatment-associated costs can range from $4000 to $9000 per patient.

[0004] Low patient compliance and adherence to the administration regimens become crucial drawbacks to the pharmacotherapy. Other major problems with current treatments are: 1) most drugs are rapidly degraded or excreted, usually within hours; 2) drugs systemically distribute throughout the body, rather than being limited to their target cells and tissues; 3) some drugs have limited bioavailability and are poorly absorbed via *e.g.* the oral route.

[0005] Within the context of the above-mentioned drawbacks associated to current therapies and the emergence of multi-drug resistant strains, a new class of drugs – antimicrobial peptides (AMPs) – arises as promising candidates for treatment of infectious diseases and TB in particular, either for administration as a monotherapy or combined with other drugs. AMPs are a diverse group of molecules belonging to the innate immune system found in most living organisms. They are commonly short in length (20-60 amino acid residues), cationic, amphipathic and have a broad spectrum against bacteria, fungi and viruses. AMPs are able to interact in both aqueous and lipid-rich environments owing to their amphipathic nature and their cationicity allows the binding to the negatively charged membranes of bacteria, causing their disruption. *In vitro* studies with the human cathelicidin LL37 have already demonstrated its involvement in the intracellular killing of *M. tuberculosis*. Noteworthy, some analogues of LL37 have been engineered to enhance its antimicrobial properties. This is the case of SEQ. ID 1 (LLKKK18), in which polar uncharged residues glutamine, asparagine and negatively charged aspartic acid are substituted by positively charged lysine. Indeed, SEQ. ID 1 (LLKKK18) has displayed higher hydrophobicity and cationicity, being three-fold more effective in the killing of mycobacteria than LL37.

[0006] In a recent publication [1] it is shown that the opportunistic strain *Mycobacterium avium* demonstrates susceptibility to direct contact with LLKKK18 (SEQ. ID 1). Indeed, this peptide reduced the growth of mycobacteria in a dose-related manner, with a concentration of 58.81 ± 0.07 μM resulting in a 50% reduction of mycobacterial growth.

[0007] However, the exogenous administration of AMPs is usually hindered due to toxicity issues, as well as limited drug stability and solubility. However, loading of AMPs in a suitable nanocarrier may help target the peptide to infected macrophages and promote the co-localization of the AMP with bacteria [2].

[0008] Recently it was developed hyaluronic acid (HA)-based self-assembling nanoparticles (NPs) [3], which are able to encapsulate low molecular weight hydrophobic molecules (like LLKKK18) via electrostatic and hydrophobic interactions between hydrophobized HA and the AMP. Hyaluronic acid is a naturally occurring glucosaminoglycan, composed of alternating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine with β-(1,4) interglycosidic linkage. In normal physiological conditions, macrophages do not internalize HA. However, it has been shown that macrophages express the CD44 receptor, one of the receptors for HA, in response to an infection, thus allowing the internalization of HA. As such, these NPs may represent a promising approach to specifically deliver drugs to infected macrophages.

[0009] A patent application was submitted by Castillo and collaborators in 2010 (WO 2011031771 A1) [4] describing compositions aiming at protein delivery for the treatment of infections, inflammatory diseases, excess growth and damaged cells or organs, including a polymer backbone, an anionic domain covalently linked to the backbone and a cationic therapeutic agent, particularly a protein, bonded or electro-statically bonded to the anionic domain of the carrier. Hyaluronic acid is given as an example of the polymer backbone. Examples of anionic groups that could be linked to the backbone included: -phosphate, -sulfate, -sulfonate and –carboxyl. The 18-mer LLKKK18 (SEQ. ID 1) analogue of LL37 is described as an example of cationic therapeutic agent. However, said composition presents some drawbacks that limit its use to specific situations: 1) since it focuses on the binding of a cationic agent to the anionic group of the backbone exclusively through electrostatic interactions; 2) given examples of composition describe the production of small nanoparticles (20-70nm in diameter), which is not ideal for the uptake by macrophages, thus hindering its use in infections where bacteria hide within those cells (*e.g.* tuberculosis); 3) also, the nature of the binding between the anionic backbone and the cationic agent (ionic interaction) may be disrupted in blood circulation, due to blood's high osmolarity. Also, the anionic blood proteins can compete or bind load molecules, taking them away from the

carriers of the composition; 4) additionally, it does not allow the binding of therapeutically relevant hydrophobic molecules, since the binding to therapeutic agents is limited to electrostatic interactions.

[0010] Given the vast potential of LL37 and its analogues, these peptides have been widely used for different applications. For example, in a patent disclosed in 2006 (US 20060275303 A1), Robert Bals and collaborators protected a pharmaceutical composition comprising a peptide with the sequence of LL37 for wound treatment or diseases in which angiogenesis is reduced, and a carrier, either liquid (examples included phosphate buffered saline, syrup or peanut oil) or solid (examples include gelatin, sucrose, lactose, pectin, among others). In 2009, Yitzchak Hillman protected methods for the treatment of diseases in general involving a cathelicidin, its analogues or fragments thereof (WO 2009010968 A2). Later, the same author specifically protected a method for the treatment of Crohn's disease or ulcerative colitis based on the non-intrarectal administration of a cathelicidin, namely LL37 or its analogues (US 8202835 B2). Mondobiotech Lab Ag also holds intellectual property for the use of LL37 as a therapeutic agent regarding prophylaxis/treatment for several diseases, including cancer, autoimmune diseases, fibrotic diseases, inflammatory diseases, neurodegenerative diseases and infectious diseases (WO 2009046847 A2). A patent held by the Regents of the University of Colorado (WO2010042534 A1) discloses antimicrobial peptides considered to have superior properties in terms of specificity, resistance to degradation and broader antimicrobial activity, as well as pharmaceutical compositions comprising said peptides, for the treatment of different infectious diseases, including mycobacterial infections.

[0011] However, all these inventions describe methods that: 1) fail to provide an efficient delivery system for those peptides; 2) do not provide protection against peptide degradability by proteases; 3) are unable to stabilize said peptides; and 4) do not solve the problem of systemic toxicity associated to the majority of antimicrobial peptides.

[0012] These limitations of the available technical solutions are addressed by the present disclosure.

General description

[0013] The current disclosure relates to a novel formulation for the sustained delivery of a therapeutic agent, preferentially an AMP, for the treatment of infectious diseases, particularly those affecting the lungs and the airways. This novel formulation comprises the exogenous administration of SEQ. ID No. 1, SEQ. ID No. 2, or SEQ. ID No. 3encapsulated in a self-assembling hyaluronic acid (HA) nanogel. This formulation may also comprise low molecular weight pharmaceuticals.

[0014] Antimicrobial peptides show many advantages over conventional antibiotics, namely:

- broad-spectrum activity (due to a more general mechanism of action);
- lowered risk of resistance acquisition;
- faster killing;
- lower dosages.

[0015] The encapsulation of AMPs in drug delivery approaches can help overcome the limitations of AMPs in terms of:

- bioavailability;
- prevention of proteolytic degradation;
- enhanced stability;
- improved patient compliance.

[0016] Hyaluronic acid particles referred in the present disclosure are suitable carriers, since their amphiphilic nature allows the self-assembling in aqueous environments. By enabling both electrostatic and hydrophobic interactions, these particles allow the co-encapsulation of other hydrophobic and positively charged molecules. Thus, HA nanoparticles provide higher internalization efficiency than other reported drug delivery systems.

[0017] An aspect of the present matter specifically relates to a composition comprising an hyaluronic acid polymer and at least an antimicrobial peptide encompassing a sequence at least 95% identical to the sequence SEQ ID NO. 1 – KEFKRIVKRIKKFLRKLV; SEQ ID NO. 2 – LLGDFFRKSKEKIGKEFKRIVQRIKDFLRLRNLVPRTES; SEQ ID NO. 3 – GLLRKGGEKIGEKLKKIGQKIKNFFQKLVPQPEQ, with a therapeutically effective amount of the

previous components, in particular preferentially 96%, 97%, 98%, 99% identical to SEQ. ID 1.

[0018] Another aspect of the present disclosure is related to the use of the composition as a pharmaceutical, a medical or a veterinary composition.

[0019] In another embodiment, the composition of the present disclosure may be an inhalable or an injectable formulation.

[0020] Another aspect of the present disclosure relates to an antimicrobial peptide comprising a sequence 95% identical to SEQ ID No. 1, or a portion / fragment of said sequence for the use in the treatment of infectious diseases like tuberculosis. In particular preferably 96%, 97%, 98%, 99% identical, more preferably an antimicrobial peptide comprising SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3.

[0021] In an embodiment the composition/compound described in the present disclosure is for the use in medicine, in particular for the use in treatment of infections in the lungs and airways in mammals; namely for the use in treatment of mycobacterial infections like tuberculosis; or for the use in treatment of other airway infections, including croup, epiglottitis, retropharyngeal abscess, pharyngitis, peritonsillar abscesses, pneumonia and bronchiolitis.

[0022] In another embodiment, the antimicrobial peptide promotes the killing of different strains of the *Mycobacterium* genera, including but not limited to, *M. tuberculosis*, *M. avium*, *M. smegmatis*, *M. bovis* and *M. marinum*.

[0023] An aspect of the present subject matter is related to a composition comprising:

an hyaluronic acid polymer loaded with at least an antimicrobial peptide, preferably the antimicrobial peptide is encapsulated or grafted into the hyaluronic acid polymer;

wherein the hyaluronic acid polymer comprises a hydrophobic chain, preferably a grafted hydrophobic chain;

wherein the antimicrobial peptide comprises a sequence at least 95% identical to one of the sequences of the following list: SEQ ID NO. 1 – KEFKRIVKRIKKFLRKLV; SEQ ID NO. 2 – LLGDFFRKSKEKIGKEFKRIVQRIKDFLRLRNLVPRTES; SEQ ID NO. 3 –

GLLRKGGEKIGEKLKKIGQKIKNFFQKLVPQPEQ, or mixtures thereof;

and wherein the composition has a therapeutically effective amount of hyaluronic acid polymer and antimicrobial peptide.

with a therapeutically effective amount of all the previous components, in particular preferably 96%, 97,%, 98%, 99% identical to SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, or mixtures thereof.

[0024] In other embodiment, the composition of the present disclosure may comprise an antimicrobial peptide comprising at least one of the sequences of the following list: SEQ ID NO. 1; SEQ ID NO. 2; SEQ ID NO. 3, or mixtures thereof.

[0025] In other embodiment, the composition of the present disclosure may further comprise an AMP comprising SEQ ID NO. 2; SEQ ID NO. 3, or mixtures thereof.

[0026] In other embodiment, the antimicrobial peptide concentration may be between 1 nM - 200 μM, preferably 10 - 150 μM, more preferably 50-100 μM.

[0027] In other embodiment, the hyaluronic acid polymer is a nanogel.

[0028] In other embodiment, the hyaluronic acid concentration is between 0.1-10 mg/ml, in particular 0.5-2 mg/ml.

[0029] In other embodiment, the hyaluronic acid polymer has a molecular weight between 1,000-200,000 g/mol.

[0030] In other embodiment, the hyaluronic acid polymer has a molecular weight between 5,000-10,000 g/mol.

[0031] In other embodiment, the hydrophobic chain comprises at least one free terminal group selected from a list consisting of: a free terminal thiol group, a free terminal methyl group, a free terminal carboxyl group, a free terminal hydroxyl group, a free terminal amino group, or mixture thereof.

[0032] In another embodiment, the composition of the present disclosure may further comprise a low molecular therapeutic agent, particularly a small hydrophobic molecule, more particularly an antibiotic.

[0033] In another embodiment, the antibiotics may include, but is not limited to, rifampicin, isoniazid, pyrazinamide, ethambutol, ethionamide, prothionamide, cycloserine, capreomycin or fluoroquinolones or their mixtures.

[0034] In another embodiment, the low molecular therapeutic agent is present at its therapeutical range which may vary between 5 to 25 mg/Kg relative to the animal's weight, depending on the low molecular weight pharmaceutical used.

[0035] The present solution is an affordable and efficient formulation to safely and efficiently deliver AMPs and low molecular weight pharmaceuticals to infected tissues, in particular lungs and airways. Therefore, this delivery system has great potential as a therapeutic approach for the treatment of tuberculosis.

[0036] Antimicrobial peptides (AMPs) are commonly small, cationic and amphipathic peptides, which play an important role in the innate immune system. LL37 – SEQ ID NO. 2, corresponding to the C-terminal of the pro-peptide hCAP18 is the only cathelicidin (a family of AMPs) found in humans, being overexpressed in several tissues and cell types. LL37 exhibits a broad antimicrobial activity by causing the membrane disruption of bacteria cell wall. Such effects occur because the amphipathic cationic α-helix peptide binds to negatively charged groups of the bacterial outer membrane. In addition to its antimicrobial activity, LL37 has also been reported as inducing mast cells/leukocytes chemotaxis, angiogenesis and wound healing. Noteworthy, LL37 shares many similarities with the murine cRAMP (cathelin-related antimicrobial peptide), including not only the α-helical conformation, but also a similar antimicrobial activity spectra and tissue distribution (Ramos R, Silva JP, Rodrigues AC, Costa R, Guardão L, Schmitt F, Gama M. (2011) Wound healing activity of the human antimicrobial peptide LL37. Peptides 32, 1469-1476). [5]

[0037] AMP administration arises as a promising strategy against mycobacteria infections, including tuberculosis. Indeed, exogenous addition of LL37 – SEQ ID NO. 2, has already been demonstrated *in vitro* to fight mycobacteria residing in macrophages. However, endogenous overexpression results in limited therapeutic effects due to poor co-localization of drug and bacteria and exogenous administration is hampered by the toxicity and limited drug stability and solubility.

[0038] Analogues of LL37 – SEQ ID NO. 2, have been engineered to enhance its antimicrobial properties, as well as to decrease cytotoxicity and plasma protein binding. One of them, LLKKK18 (SEQ ID NO. 1), has a backbone similar to LL37 – SEQ ID NO. 2, but is composed of only 18 amino acids. Also, the polar uncharged residues glutamine and asparagine, as well as the negatively charged aspartic acid, are substituted by positively charged lysine residues. This peptide shows higher cationicity and hydrophobicity compared to LL37, which is important to attain higher antimicrobial and chemoattractant activities. Also, due to its smaller size, LLKKK18 (SEQ. ID NO. 1) can be transported across cell membranes more easily and its synthesis is less expensive.

[0039] The disclosure described herein also relates to a novel formulation for intra-tracheal or intravenous administration of an antimicrobial peptide for the treatment of infections of the lungs and airways, in particular tuberculosis. Said formulation is based on the encapsulation of an AMP, a low molecular weight pharmaceutical, or mixtures thereof in self-assembling hydrophobized hyaluronic acid nanoparticles.

[0040] In an embodiment, the HA nanogel facilitates the targeting of the therapeutic agent to activated macrophages since these express the CD44 receptor, which binds HA, thus enhancing its internalization.

[0041] In an embodiment, the antimicrobial peptide concentration is between 1 nM -200 μM, in particular 10 - 200 μM.

[0042] In an embodiment, it is shown that the (SEQ. ID NO. 1) LLKKK18-loaded HA nanoparticles are preferable not toxic to bone marrow-derived macrophages up to an antimicrobial peptide (SEQ. ID. NO. 1) concentration of 100 μM.

[0043] In an embodiment, it is shown that SEQ ID NO. 1(LLKKK18) significantly reduces the growth of *M. tuberculosis* in an axenic culture.

[0044] In an embodiment, it is shown that SEQ ID NO. 1(LLKKK18) loaded HA nanoparticles are internalized by marrow-derived macrophages, in a time-frame of 7 hours.

[0045] In an embodiment, it is shown that bone marrow-derived macrophages infected *in vitro* with *Mycobacterium avium* or *M. tuberculosis* present a significantly lower

mycobacteria load, after treatment with SEQ ID NO. 1 (LLKKK18).

[0046] In another embodiment, it is shown that antimicrobial peptide (SEQ ID NO. 1)-loaded HA nanoparticles significantly reduces the mycobacterial load of mice infected with either *Mycobacterium avium* or *Mycobacterium tuberculosis*. A solution of phosphate buffered saline (PBS) was used as control. As depicted in Fig. 6, the intra-tracheal administration of these SEQ ID NO. 1 (LLKKK18) -loaded nanoparticles carried out over a period of 10 days (5 administrations in total) significantly reduced the infection levels in C57BL/6 mice infected with *M. tuberculosis* for a 3-month period. This is indicative of the involvement of this peptide in ameliorating mycobacterial infections.

[0047] The present disclosure also relates to a formulation for improvement of infetions of the lungs and airways, consisting on the delivery of an antimicrobial peptide, preferably SEQ ID NO. 1 (LLKKK18), wherein said formulation stabilizes said peptide, prevents its degradation by proteases and allows its sustained release.

[0048] In an embodiment, said AMP is SEQ ID NO. 1 (LLKKK18).

[0049] In an embodiment, the hyaluronic acid has a molecular weight between 1,000-200,000 g/mol, preferably 1,000-10,000 g/mol, more preferably between 5,000-10,000 g/mol.

[0050] In an embodiment, the therapeutic agent is loaded/encapsulated via immersion in an aqueous solution containing hyaluronic acid particles.

[0051] In an embodiment, a hydrophobic chain is grafted to said hyaluronic acid particles via an amide bond formation. The introduction of said hydrophobic graft is important for the self-assembling of the nanoparticles in aqueous solutions, thus enhancing the encapsulation of hydrophobic molecules.

[0052] In another embodiment, said hydrophobic chain may contain a free terminal thiol group, a free terminal methyl group, a free terminal carboxyl group, a free terminal hydroxyl group, a free terminal amino group, or mixture thereof. Preferably a free terminal thiol group, more preferably an 11- amino-undecanethiol, among other.

[0053] In an embodiment, hyaluronic acid particles concentration ranges between 0.1 and 10 mg/ml, preferable 0.5 and 2 mg/ml.

[0054] The present disclosure also relates to a method for treating infectious diseases, particularly of the lungs and airways, in particular tuberculosis, which comprises the administration of the formulation to a mammal in need.

[0055] In an embodiment, said method is a method of treating upper or lower airway infections in a mammal in need thereof, in particular, said mammal is human, equine, canine, feline, porcine or any other domestic or agricultural species.

[0056] In an embodiment, the AMP is administered in combination with any another compound.

[0057] In an embodiment, the AMP-loaded nanoparticles are administered intra-tracheally. In another embodiment, said nanoparticles are administered to the deep lung in an aerosolized formulation. In another embodiment, said nanoparticles are administered intravenously, intra-peritoneally or sub-cutaneously.

[0058] Preferred routes of administration of the AMP-loaded nanoparticles include but are not limited to oral, parenteral, intramuscular, intravenous, in situ injection, intranasal, sublingual, intratracheal, inhalation or topical.

[0059] In some embodiments, the AMP-loaded nanoparticles dose or dosage may be administered to the subject, for example, once a day, twice a day, or three times a day. In other embodiments, the dose is administered to the subject once a week, once a month, once every two months, four times a year, three times a year, twice a year, or once a year.

[0060] In an embodiment, the disclosed AMP-containing delivery system is a novel, safe, non-expensive and effective approach to address treatment of mycobacterial infections. This was achieved through a reduction in the mycobacterial load in infected macrophages present in the lungs, after intra-tracheal delivery. Therefore, this delivery system has great potential as a therapeutic approach for tuberculosis treatment.

[0061] Methods for the alignment of sequences for comparison are well known in the art, such methods include GAP, BESTFIT, BLAST, FASTA and TFASTA. GAP uses the algorithm of Needleman and Wunsch ((1970) J Mol Biol 48: 443-453) to find the global (over the whole the sequence) alignment of two sequences that maximizes the number of matches and minimizes the number of gaps. The BLAST algorithm (Altschul et al. (1990) J Mol Biol 215:

403-10) calculates percent sequence identity and performs a statistical analysis of the similarity between the two sequences. The software for performing BLAST analysis is publicly available through the National Centre for Biotechnology Information (NCBI). Global percentages of similarity and identity may also be determined using one of the methods available in the MatGAT software package (Campanella et al., BMC Bioinformatics. 2003 Jul 10; 4:29. MatGAT: an application that generates similarity/identity matrices using protein or DNA sequences). Minor manual editing may be performed to optimise alignment between conserved motifs, as would be apparent to a person skilled in the art. The sequence identity values, which are indicated in the present subject matter as a percentage were determined over the entire amino acid sequence, using BLAST with the default parameters.

[0062] Throughout the description and claims the word "comprise" and variations of the word, are not intended to exclude other technical features, additives, components, or steps. Additional objects, advantages and features of the disclosure will become apparent to those skilled in the art upon examination of the description or may be learned by practice of the disclosure. The following examples and drawings are provided by way of illustration and are not intended to limit the present disclosure. Furthermore, the present disclosure covers all possible combinations of particular and preferred embodiments described herein.

Brief description of the drawings

[0063] **Fig. 1** – Characterization of the AMP-loaded formulations. Average size (A) and Zeta potential (B) after encapsulation of 100 µM LLKKK18 (SEQ. ID 1) in a 0.5 mg/ml water dispersion of the HA nanogel. After overnight incubation, formulations were filtered through a pore size of 0.22 µm. Both measurements were carried out in a Malvern Zetasizer. Values represent mean ± SD value (n=6). *** $p < 0.001$, compared to 0.5 mg/ml HA.

[0064] **Fig. 2** – Cytotoxic activity of encapsulated LLKKK18 (SEQ. ID 1) on BMMϕ tested with Trypan blue (A) and Live/Dead (B) after 24h of incubation. The percentage of viable cells after treatment was calculated relatively to the untreated group. Values represent mean ± SEM value (n=3). * $p < 0.05$, *** $p < 0.001$, compared to 0.5 mg/ml HA.

[0065] **Fig. 3** - Dose-response curve of the inhibition of *M. tuberculosis* growth against different peptide concentrations, after 4 days of incubation. The IC_{50} values were calculated

from the represented curve, as the concentration needed to inhibit by 50% the mycobacterial growth. The mean ± SD is represented for each concentration considering the results obtained in triplicates.

[0066] **Fig. 4** – Internalization kinetics for the LLKKK18 (SEQ. NO. ID 1)-loaded HA nanoparticles. BMMϕ cells were incubated with 100 μM of TAMRA (red)-bound LLKKK18 (SEQ. NO. ID 1) encapsulated in 0.5 mg/ml HA and its internalization followed over time using a confocal microscope. BMMϕ nuclei were stained with DAPI (blue).

[0067] **Fig. 5** - Intracellular killing of *M. avium* (A) and *M. tuberculosis* (B) in infected BMMϕ, promoted by AMP-loaded HA nanoparticles. Data points were obtained in triplicates and show the mean ± SEM value for at least 3 independent experiments. * $p < 0.05$, compared to control.

[0068] **Fig. 6** – *In vivo* killing of either *M. avium* strain 2447 (A), *M. avium* strain 25291 (B) or *M. tuberculosis* (C) promoted by HA-loaded LLKKK18 (SEQ. ID NO. 1). C57BL/6 mice were infected with each one of *Mycobacterium* strains via the pulmonary route. After 3 months, five or ten doses of the treatments (50 μl) were administered intra-tracheally using a MicroSprayer® aerosoliser, every other day. In the mice infected with *M. tuberculosis* an antibiotic cocktail containing rifampicin, pyrazinamide and isoniazid was used as a standard treatment. Data represents the mean ± SD for at least 6 mice per group. *$p < 0.05$, ***$p < 0.001$, compared to control, with respective number of administrations. $^{+}p < 0.05$, $^{+++}p < 0.001$, compared to 0.5 mg/ml HA, with respective number of administrations.

Detailed description

[0069] In an embodiment, the production and characterization of HA-based nanogels was performed according to the procedure previously described by Pedrosa and co-workers (2014) [3]. Briefly, cations belonging to sodium hyaluronic acid (Lifecore Biomedical, 7.46 kDa, 401 g/mol per dissacharide unit) were first exchanged with the lipophilic tetrabutylammonium (TBA) ion to enable HA solubilization in dimethyl sulfoxide (DMSO), using a cationic exchange resin (AG 50W). The resin was separated by centrifugation and the supernatant (HA-TBA) was frozen at -80 °C until freeze-drying. A 11- amino-undecanethiol

(AT) hydrophobic chain was then grafted to HA-TBA through amide formation. The final solution was first dialyzed against NaCl (150 mM) to remove remaining TBA ions and subsequently against distilled water to remove NaCl. The final solution containing HA-AT nanogel was frozen at -80 °C and freeze-dried for proper storage.

[0070] In an embodiment, the encapsulation of the antimicrobial peptide into HA-based nanogels was carried out as follows: LLKKK18 (SEQ. ID 1) was loaded into the nanogel by dissolving them together in Phosphate Buffered Saline (PBS) or cell culture medium without serum for approximately 24h at room temperature, in a spinning wheel. Free peptide was removed using centrifugal filter tubes with a molecular weight cut-off of 10 KDa (Millipore), which do not allow the nanogel to pass through. The loaded nanogel, concentrated in the centricon, was then diluted to the original volume with PBS or cell culture medium without serum and sterilized by filtration using a 0.22 μm membrane filter of low protein binding Polyethersulfone (PES). Formulations were stored at 4 °C until used.

[0071] HA nanogel characterization (Size distribution and Zeta Potential)

[0072] In an embodiment, the HA nanogel characterization (Size distribution and Zeta Potential) was carried out as follows: since these HA nanogels are self-assembled colloidal nanocarriers, their physical properties can get significantly modified once bioactive molecules have been successfully entrapped within the inner hydrophobic core structure. Thus, the characterization of the HA nanogel was carried out for the empty and encapsulated nanogel. The size distribution and zeta potential measurements for each colloidal dispersion, prepared as previously described and at the range of concentrations used for the *in vitro*/*in vivo* tests (100 μM), were performed in a Malvern Zetasizer NANO ZS (Malvern Instruments Limited, UK) at room temperature. For each sample (1 mL – dispersed in miliQ water), both *z*-average diameter, which corresponds to the mean hydrodynamic diameter, and zeta potential, were evaluated after 6 repeated measurements using either a polystyrene cell (size determination) or a folded capillary cell (zeta potential). Both empty and encapsulated nanogel samples were filtered (pore size 0.22 μm) prior to the measurements.

[0073] In an embodiment and in agreement with previous data [3], the HA-based composition alone forms nanoparticles of 133 ± 4 nm (PDI=0.27±0.02) (**Fig. 1**). Moreover, due to the natural negative charge of the linear polymer of HA, the nanogel itself presents a

negative surface charge that provides good stability properties to the colloidal system as justified by a zeta potential of -23±1 mV. These two characteristics – size and zeta potential – significantly and surprisingly changed when positively charged AMPs, in particular LLKKK18 shown as an example, were added to the system. It is also possible that in addition to LLKKK18's cationicity, its hydrophobicity may help promote a higher internalization.

[0074] In an embodiment and according to this result, the overall change in charge contributes to size increments up to 553 ± 13 nm (PDI=0.1±0.1), which could be possibly related with the aggregation of the NPs. Moreover, peptide internalization into the nanogel contributes to narrowing down monomodal size distributions as polydispersity index's (PDI) get smaller when compared to empty nanogel. The ultimate goal of the drug delivery system being designed is to deliver AMPs into the intracellular space of infected cells. Accordingly, the overall effect of encapsulated nanogels has a positive effect in terms of promoting its uptake by macrophages. Indeed, macrophages preferentially internalize particles over 500 nm. Thus, the AMP-formulations now disclosed, for example in Fig. 1A, are surprisingly adequate to be internalized by macrophages. Also, the negatively charged cell surfaces of macrophages are able to recognize and attract more positively charged particles.

[0075] In an embodiment, the culture of mouse bone marrow-derived macrophages was carried out as follows: bone marrow cells from C57BL/6 animals were isolated from femurs and tibia by flushing the bone with culture media and differentiating them into mouse macrophages (BMMϕ) in the presence of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 20% L-cell conditioned medium (LCCM), which contains macrophage colony stimulating factor (M-CSF). Cells were grown and differentiated in large Petri dishes until day 7 when adherent/differentiated cells were mechanically removed using a cell scraper and seeded at the desired concentration in multi-well plates. By using this procedure we assure that all the cells used during the experiment were differentiated.

[0076] In an embodiment, the trypan blue assay was carried out as follows: BMMϕ were seeded at 5×10^4 cells/well in a 96-well plate and left to adhere overnight at 37 °C in a humidified atmosphere of 5% CO_2. Cells were then incubated with either 0.5 mg/ml HA or 0.5 mg/ml HA + 100 µM LLKKK18. After 24h, the plate was placed for 1h on ice to detach BMMϕ, which were then mechanically collected using a micropipette tip and diluted 1:1 with

Trypan Blue for cell counting in a Neubauer chamber. Blue-stained cells were considered as dead cells (membrane disruption allows the internalization of the dye) and cell viability reflects the balance between dead and live cells for each group tested. An untreated group was used as a control.

[0077] In an embodiment, live/dead assay were performed. LIVE/DEAD® Viability/Cytotoxicity Kit (Invitrogen, CA) was used to determine cell viability after BMMϕ treatment with either the HA nanogel or the AMP-loaded nanogel. This assay is based on the use of two fluorescent probes, calcein-AM and ethidium homodimer-1, which allows the simultaneous determination of live and dead cells with two probes, by evaluating intracellular esterase activity and plasma membrane integrity, respectively. Briefly, cell culture medium in each well was removed and immediately replaced with 100 μl of PBS containing 1 μM calcein AM and 2 μM ethidium homodimer-1. Cells were then incubated for 45 min at 37 °C, 5% CO_2 and visualized in a fluorescence microscope.

[0078] As depicted in Fig. 2, the AMP-loaded nanogel causes no toxicity to BMMϕ up to a concentration of 100 μM LLKKK18 (SEQ. ID 1), as demonstrated with Trypan Blue and Live/Dead assays. This is indicative that at concentrations up to 100 μM, the peptide can be administered without causing the death of the healthy macrophages. At a 200 μM concentration, some cytotoxicity is observed.

[0079] In an embodiment, the effect of LLKKK18 (SEQ. ID 1) on the growth of *M. tuberculosis* was performed. Aliquots of *M. tuberculosis* H37Rv were diluted in 7H9 broth medium to attain a concentration of 2×10^4 CFU/mL and the assay was initiated by adding 50 μL of axenic culture to each well of a 96-well plate. The mycobactericidal effect of LLKKK18 (SEQ. ID 1) was determined by adding sterile serial dilutions, in particular between 25 and 100 μM of the peptide dissolved in distilled water to the axenic culture. Total volume per well was kept at 100 μL. Sterile distilled water was used as a control. The microplate was then incubated in a humidified chamber at 37 °C during 4 days after which the antimicrobial effect was evaluated by plating serial dilutions of the suspensions on solid Middlebrook 7H11 agar medium supplemented with 10% OADC. CFUs in each plate were counted 3 weeks after plating. Mycobacterial survival after 4 days of incubation was expressed as a percentage towards a control group of mycobacteria grown in the absence of AMP.

[0080] In an embodiment, results showed that after 4 days of incubation LLKKK18 (SEQ. ID 1) decreased the growth of *M. tuberculosis* in a dose-dependent manner (**Fig. 3**), with a concentration around 50 µM leading to a 50% reduction in the mycobacterial growth.

[0081] In an embodiment, internalization studies were performed. BMMϕ were differentiated as previously described and seeded at $5{\times}10^5$ cells/well in 24-well plates containing coverslips. LLKKK18 (SEQ. ID 1) was labelled with the fluorescent tag TAMRA prior to loading. BMMϕ were then incubated in the presence of TAMRA-LLKKK18 (SEQ. ID NO. 1) -loaded NPs at 37 °C, 5% CO_2, and its internalization followed over time. Encapsulated LLKKK18 (SEQ. ID 1) was added at 30 µM per well in 0.5 $mg.ml^{-1}$ of HA nanogel. At different time points, cells were fixed with 2% paraformaldehyde during 20 min and the coverslips visualized using a confocal microscope.

[0082] In an embodiment, data obtained shows that LLKKK18 (SEQ. NO. ID 1) is internalized within few hours (**Fig. 4**). The amount of internalized LLKKK18 (SEQ. NO. ID 1) increased up to 7h, as indicated by an increase in TAMRA-labelled area around the macrophages nuclei (stained with DAPI).

[0083] In an embodiment, *in vitro* intracellular killing assay were carried out._To examine whether AMP-loaded NPs reduce bacterial burden within macrophages, $5{\times}10^5$ cells (BMMϕ) were infected with *M. tuberculosis* H37Rv for 4h at a multiplicity of infection of 2. Non-internalized bacteria was removed by washing each well four times with pre-warmed DMEM and treatments, in particular control, HA and HA+LLKKK18, were then applied and cells were then incubated for 4 days at 37 °C, 5% CO_2. Cells were permeabilized with saponin and the intracellular survival was evaluated by plating serial dilutions on 7H11 plates. CFUs counting was performed after 3 weeks of incubation at 37 °C, 5% CO_2. The initial intracellular bacterial burden was assessed identically at day 0.

[0084] As observed in **Fig. 5**, the incubation of mouse bone marrow-derived macrophages with LLKKK18-loaded HA NPs led to a significant reduction of either *M. avium* (**Fig. 5A**) or *M. tuberculosis* (**Fig. 5B**) within the macrophages. Whereas in the case of *M. avium* this reached a ~3-log reduction, an approximately 2-log decrease was observed with *M. tuberculosis*. This difference may be explained by the higher virulence of *M. tuberculosis*, mostly likely due to the different composition of the cell membrane.

[0085] In an embodiment, animal infection and intra-tracheal treatments were carried out. 6-8 weeks old female C57BL/6 mice, obtained from Charles River (Barcelona, Spain) were used in this study. Mice were infected aerogenically by exposure to an aerosolized suspension of either *M. avium* strain 2447, *M. avium* strain 25291 or *M. tuberculosis* strain HRv37 during 40 min using a Glas-Col Inhalation Exposure System: a 2×10^6 CFU/ml suspension resulted in the implantation of ~20 CFU of mycobacteria in the lungs of each mouse. The infection was allowed to proliferate *in vivo* during 3 months, after which mice were individually anesthetized with Ketamine/Medetomidine and treated 5 times every other day. Treatments were performed by injecting 50 µL of the therapeutic formulations directly into the trachea, using a MicroSprayer® aerosoliser (IA-1C; Penn-Century, Philadelphia, PA, USA) attached to a high-pressure syringe (FMJ-250; Penn-Century). As control groups both PBS and unloaded HA nanogel (0.5 mg/ml) were used. Encapsulated LLKKK18 (SEQ. ID 1) was applied at the concentration of 100 µM. A group of *M. avium* strain 25291-infected animals was treated with a cocktail of 0.4 mg/ml rifampicin, 1.94 mg/ml clarithromycin and 0.25 mg/ml ethambutol as a standard antibiotic treatment. Similarly, a group of *M. tuberculosis*-infected animals was treated with a cocktail of 10 mg/Kg rifampicin, 150 mg/Kg pyrazinamide and 25 mg/Kg isoniazid as a standard antibiotic treatment.

[0086] In an embodiment, seven days after the last treatment, mice were sacrificed by CO_2 asphyxiation and lungs were aseptically excised and homogenized in DMEM. A pre-treatment with collagenase (37 °C, 30 min) was applied to easily degrade extracellular matrix and saponin was added to the lung homogenate to release intracellular mycobacteria. Serial dilutions were plated on 7H11 agar and CFUs were counted after 7 to 10 days of incubation at 37 °C.

[0087] In an embodiment, all animal experiments were performed according to the European Union Directive 86/609/EEC and were approved by the Portuguese Veterinary Authorities.

[0088] In an embodiment, as shown in **Fig. 6**, the AMP-loaded HA NPs induce a statistically significant reduction of mycobacterial load (a total of ~1 log compared to PBS) of either *M. avium* strain 2447 (**Fig. 6A**), *M. avium* strain 25291 (**Fig. 6B**) or *M. tuberculosis* (**Fig. 6C**) after a 5-administration treatment every other day. In the particular case of *M. avium* strain

25291, all treatments led to a significant reduction of mycobacteria growth, but no differences were found among different treatments. Noteworthy, even the antibiotic cocktail did not induce a high decrease of mycobacterial load. Also, the decrease in mycobacteria numbers observed with the AMP-loaded NPs was similar to the one observed for a cocktail of antibiotics (rifampicin, isoniazid and ethambutol. Also, treatment regimens, as well as routes of administration, were different: 5 consecutive treatments by oral gavage for the antibiotic cocktail vs 5 every other day intra-tracheal applications for the NPs. It should be taken into consideration that for the microsprayer application, it is necessary to anesthetize the mice, which is a procedure not recommended performing every day.

[0089] Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. The scope of the present invention is not intended to be limited to the above description, but rather is as set forth in the appended claims.

[0090] Where singular forms of elements or features are used in the specification of the claims, the plural form is also included, and vice versa, if not specifically excluded. For example, the term "a cell" or "the cell" also includes the plural forms "cells" or "the cells," and vice versa. In the claims articles such as "a," "an," and "the" may mean one or more than one unless indicated to the contrary or otherwise evident from the context. Claims or descriptions that include "or" between one or more members of a group are considered satisfied if one, more than one, or all of the group members are present in, employed in, or otherwise relevant to a given product or process unless indicated to the contrary or otherwise evident from the context. The invention includes embodiments in which exactly one member of the group is present in, employed in, or otherwise relevant to a given product or process. The invention also includes embodiments in which more than one, or all of the group members are present in, employed in, or otherwise relevant to a given product or process.

[0091] Furthermore, it is to be understood that the disclosure encompasses all variations, combinations, and permutations in which one or more limitations, elements, clauses, descriptive terms, etc., from one or more of the claims or from relevant portions of the description is introduced into another claim. For example, any claim that is dependent on another claim can be modified to include one or more limitations found in any other claim

that is dependent on the same base claim. Furthermore, where the claims recite a composition, it is to be understood that methods of using the composition for any of the purposes disclosed herein are included, and methods of making the composition according to any of the methods of making disclosed herein or other methods known in the art are included, unless otherwise indicated or unless it would be evident to one of ordinary skill in the art that a contradiction or inconsistency would arise.

[0092] Where ranges are given, endpoints are included. Furthermore, it is to be understood that unless otherwise indicated or otherwise evident from the context and/or the understanding of one of ordinary skill in the art, values that are expressed as ranges can assume any specific value within the stated ranges in different embodiments of the invention, to the tenth of the unit of the lower limit of the range, unless the context clearly dictates otherwise. It is also to be understood that unless otherwise indicated or otherwise evident from the context and/or the understanding of one of ordinary skill in the art, values expressed as ranges can assume any subrange within the given range, wherein the endpoints of the subrange are expressed to the same degree of accuracy as the tenth of the unit of the lower limit of the range.

[0093] In addition, it is to be understood that any particular embodiment of the present invention may be explicitly excluded from any one or more of the claims. Where ranges are given, any value within the range may explicitly be excluded from any one or more of the claims. Any embodiment, element, feature, application, or aspect of the compositions and/or methods of the invention, can be excluded from any one or more claims. For purposes of brevity, all of the embodiments in which one or more elements, features, purposes, or aspects are excluded are not set forth explicitly herein.

[0094] The above described embodiments are combinable.

[0095] The following claims further set out particular embodiments of the disclosure.

[0096] All references recited in this document are incorporated herein in their entirety by reference, as if each and every reference had been incorporated by reference individually.

References:

1. Santos JC, Silva-Gomes S, Silva JP, Gama FM, Rosa G, Gallo RL, Appelberg R. (2014) Endogenous cathelicidin production limits inflammation and protective immunity to Mycobacterium avium in mice. Immunity, Inflammation and Disease 2, 1-12.

2. Sonawane A, Santos JC, Mishra B, Jena P, Progida C, Sorensen OE, Gallo R, Appelberg R, Griffiths G. (2011) Cathelicidin is involved in the intra-cellular killing of mycobacteria in macrophages. Cellular Microbiology 13, 1601-1617.

3. Pedrosa SS, Goncalves C, David L, Gama M. (2014) A novel crosslinked hyaluronic Acid nanogel for drug delivery. Macromol Biosci 14, 1556-68.

4. Castillo GM, Bolotin EM, Akiko N-A. (2012) Anionic-core composition for delivery of therapeutic agents, and methods of making and using the same Journal WO 2011031771 A1,

5. Ramos R, Silva JP, Rodrigues AC, Costa R, Guardão L, Schmitt F, Soares R, Vilanova M, Domingues L, Gama M. (2011) Wound healing activity of the human antimicrobial peptide LL37. Peptides 32, 1469-1476.

C L A I M S

1. A composition comprising:
an hyaluronic acid polymer loaded with at least an antimicrobial peptide,
wherein the hyaluronic acid polymer comprises a hydrophobic chain,
wherein the antimicrobial peptide comprises a sequence at least 95% identical to one of the sequences of the following list: SEQ ID NO. 1; SEQ ID NO. 2; SEQ ID NO. 3;
and wherein the composition has a therapeutically effective amount of hyaluronic acid polymer and antimicrobial peptide.

2. The composition according to the previous claim wherein the sequence is at least 96%, 97%, 98% or 99% identical to SEQ ID NO. 1; SEQ ID NO. 2; SEQ ID NO. 3, or mixtures thereof.

3. The composition according to the previous claims wherein the antimicrobial peptide is encapsulated or grafted into the hyaluronic acid polymer.

4. The composition according to any of the previous claims wherein the antimicrobial peptide concentration is between 1 nM - 200 μM, preferably 10 - 150 μM, more preferably 50-100 μM.

5. The composition according to any one of the previous claims wherein the hyaluronic acid polymer is a nanogel.

6. The composition according to any one of the previous claims wherein said hyaluronic acid polymer concentration is between 0.1-10 mg/ml, in particular 0.5-2 mg/ml.

7. The composition according to any one of the previous claims wherein said hyaluronic acid polymer has a molecular weight between 1,000-200,000 g/mol.

8. The composition according to any one of the previous claims wherein said hyaluronic acid polymer has a molecular weight between 5,000-10,000 g/mol.

9. The composition according to any one of the previous claims wherein said hydrophobic chain comprises at least one free terminal group selected from a list consisting of: a free terminal thiol group, a free terminal methyl group, a free terminal carboxyl group, a free terminal hydroxyl group, a free terminal amino group, or mixture thereof.

10. The composition according to any one of the previous claims, wherein the composition is an inhalable formulation or an injectable formulation.

11. The composition according to any one of the previous claims further comprising a low molecular therapeutical agent.

12. The composition according to previous claim wherein the low molecular therapeutical agent is selected from an antibiotic, an anti-inflammatory agent, an antiseptic agent, an antipyretic agent, an hydrophobic molecule, a therapeutic agent, or mixtures thereof.

13. The composition according to previous claims 11-12, wherein the low molecular therapeutic agent is rifampicin, isoniazid, pyrazinamide, ethambutol, ethionamide, prothionamide, cycloserine, capreomycin, fluoroquinolones or mixtures thereof.

14. The composition according to any of the previous claims for use in medicine or veterinary.

15. The composition according to any of the previous claims for use in the treatment or prevention of infectious diseases, in particular in the treatment of mammal infectious diseases.

16. The composition according to the previous claim for use in the treatment or prevention of mycobacterial infections and/or infectious diseases in the lungs and/or infectious diseases in the airways.

17. The composition according to the previous claim wherein the mycobacterial infections are tuberculosis.

18. The composition according to claim 1617 wherein the infection diseases in the airways are selected from the following list: croup, epiglottitis, retropharyngeal abscess, pharyngitis, peritonsillar abscesses, pneumonia and/or bronchiolitis.

19. An antimicrobial peptide comprising a sequence at least 95% identical to SEQ ID No. 1, or a portion / fragment of said sequence for use in the treatment of infectious diseases, in particular in the treatment of mammal infectious diseases.

20. The antimicrobial peptide according to the previous claim wherein the sequence is at least 96%, 97%, 98% or 99% identical to SEQ ID NO. 1.

21. The antimicrobial peptide according to the previous claim for use in the treatment of mycobacterial infections and/or infectious diseases in the lungs and/or infectious diseases in the airways.

22. The antimicrobial peptide according to the previous claim wherein the mycobacterial infections are tuberculosis.

23. The antimicrobial peptide according to claim 22 wherein the infection diseases in the airways are selected from the following list: croup, epiglottitis, retropharyngeal abscess, pharyngitis, peritonsillar abscesses, pneumonia and/or bronchiolitis.

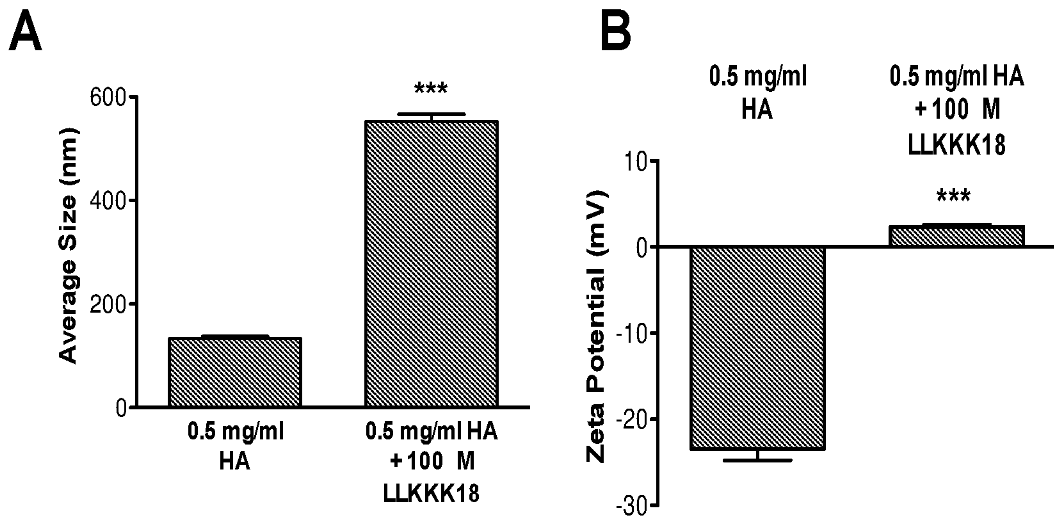

Fig. 1

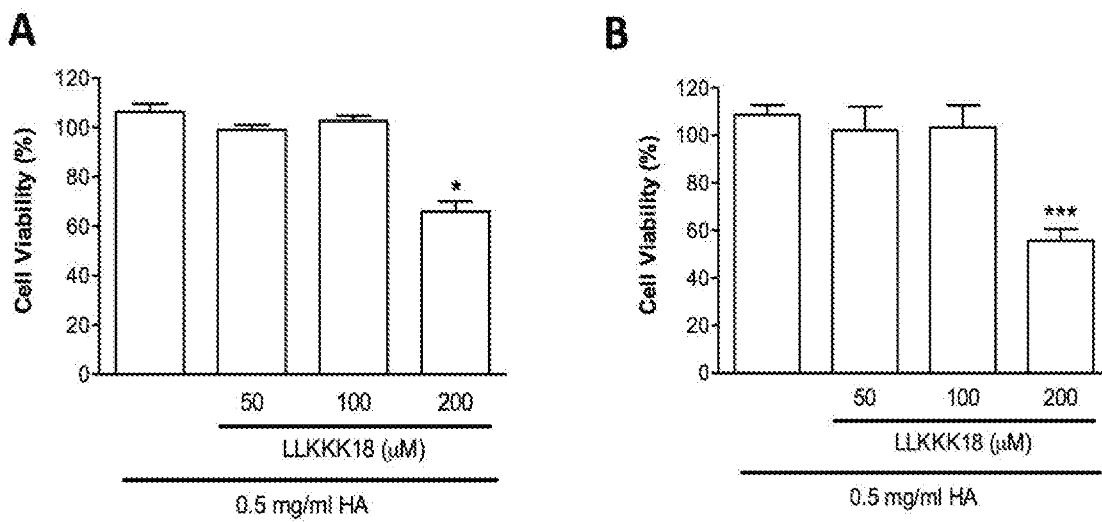

Fig. 2

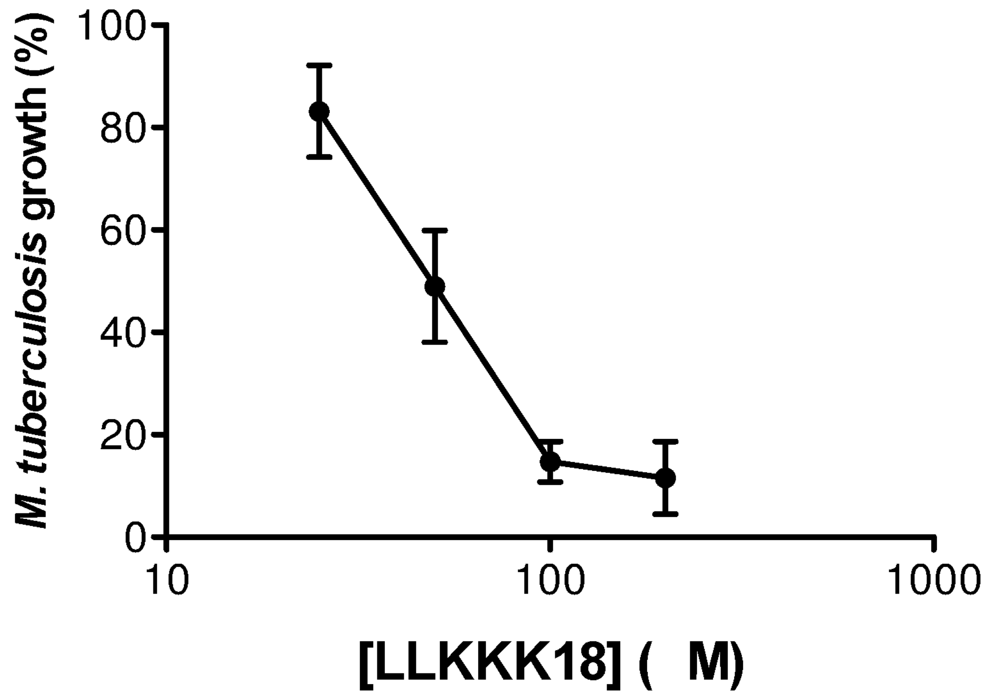

Fig. 3

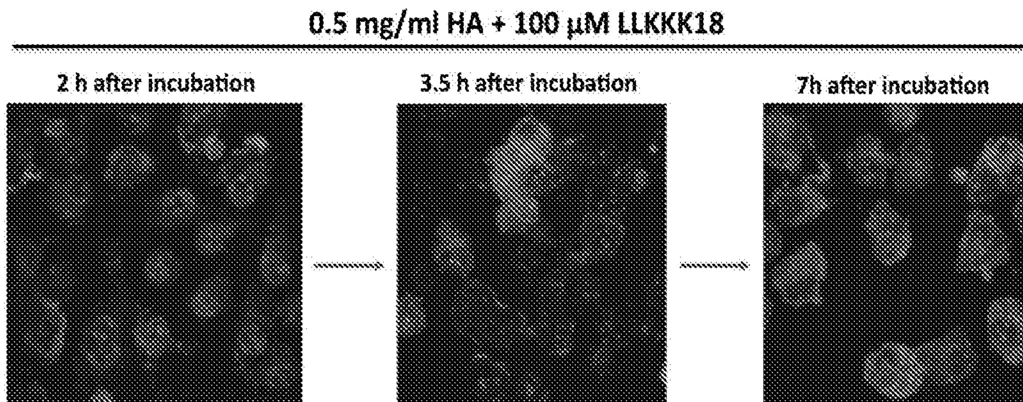

Fig. 4

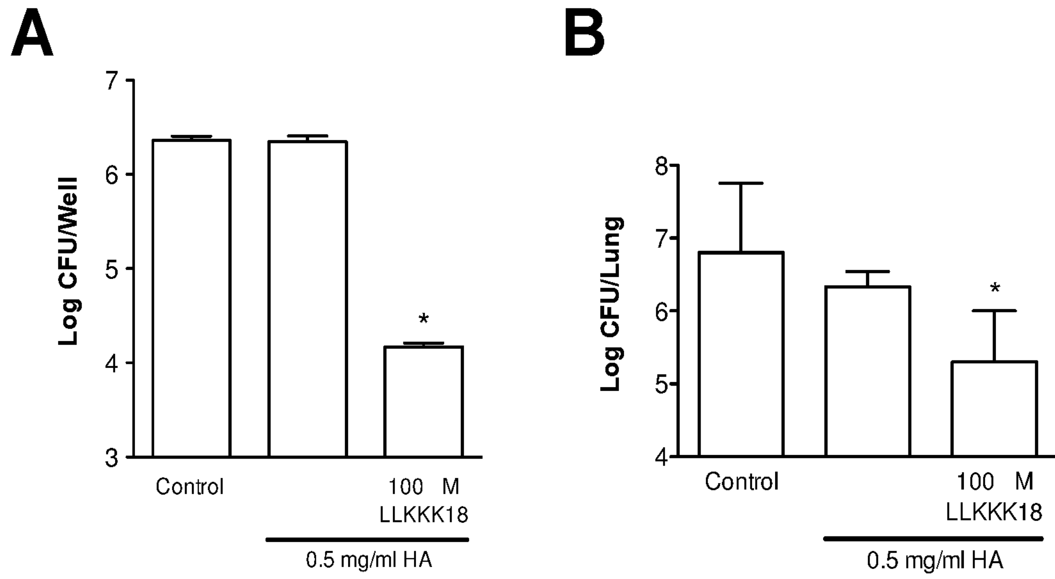

Fig. 5

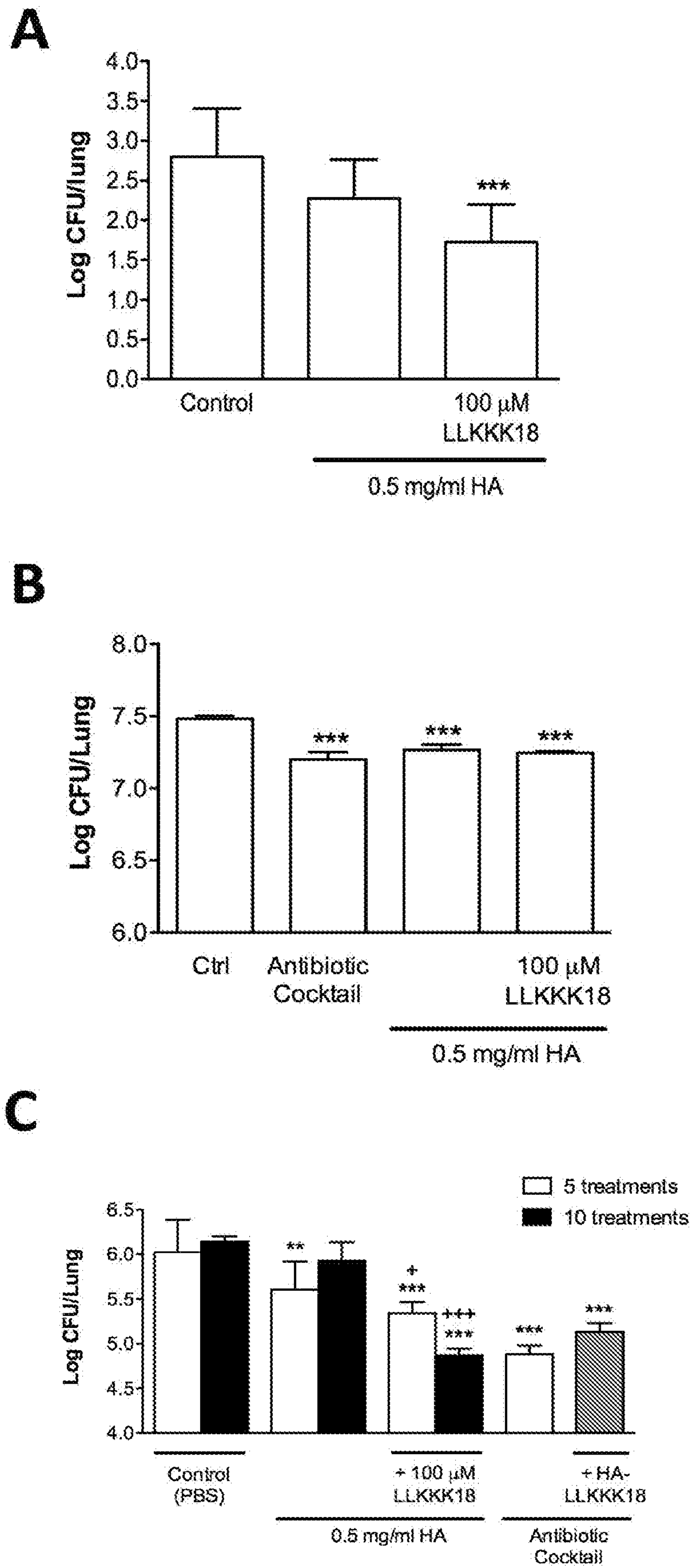

Fig. 6

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2016/057508

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K9/00 A61K47/36 A61K31/132
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 2 821 077 A1 (PRAXIS BIOPHARMA RES INST [ES]) 7 January 2015 (2015-01-07) paragraph [0040] - paragraph [0056] claims 5, 8 sequence 1	1,3-5, 14,15
X	US 2015/343014 A1 (LEFEVRE JEAN-MARIE [FR] ET AL) 3 December 2015 (2015-12-03) paragraph [0001] - paragraph [0054] claims 1-13	1-18
X	US 6 040 291 A (HIRATA MICHIMASA [JP]) 21 March 2000 (2000-03-21) column 1, line 7 - column 3, line 32 tables 1, 5 claims 1-8; example 1	19-23

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 March 2017	24/03/2017

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	González Ferreiro, M

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IB2016/057508

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 2821077 A1	07-01-2015	CN 105611916 A	25-05-2016
		EP 2821077 A1	07-01-2015
		EP 3023105 A2	25-05-2016
		JP 2016523884 A	12-08-2016
		US 2016199447 A1	14-07-2016
		WO 2015001163 A2	08-01-2015
US 2015343014 A1	03-12-2015	AR 094976 A1	09-09-2015
		CA 2897518 A1	04-09-2014
		EP 2961481 A1	06-01-2016
		FR 3002452 A1	29-08-2014
		JP 2016510017 A	04-04-2016
		KR 20150134325 A	01-12-2015
		TN 2015000282 A1	03-10-2016
		US 2015343014 A1	03-12-2015
		WO 2014131974 A1	04-09-2014
US 6040291 A	21-03-2000	AT 332922 T	15-08-2006
		CA 2264258 A1	25-09-1999
		DE 69932281 T2	12-07-2007
		EP 0955312 A2	10-11-1999
		JP 4037525 B2	23-01-2008
		JP H11335396 A	07-12-1999
		US 6040291 A	21-03-2000