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(54) Title: A PHARMACEUTICAL COMPOSITION COMPRISING AN IONOPHORE AND A BETA-LACTAM ANTIBIOTIC AND USE THEREOF

(57) Abstract: A combination comprising an ionophore and a β -lactam antibiotic is provided, as well as methods of use thereof in inhibiting Mycobacterium tuberculosis complex strains multiplication or in treating tuberculosis.

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A pharmaceutical composition comprising an ionophore and a beta-lactam antibiotic
and use thereof

FIELD OF THE INVENTION

5 The present invention relates to a combination of ionophore and β -lactam antibiotic and as well as methods of use thereof in treating tuberculosis, especially in inhibiting the development of the tuberculosis disease.

BACKGROUND OF THE INVENTION

10 Tuberculosis is primarily a disease of the lung and is characterized by chronic coughing, blood-tinged sputum, fever, night sweats, and a loss of appetite. Among infectious diseases, tuberculosis (TB) remains a major public health problem. According to the report of the World Health Organization, about 10 million cases occur each year, among which 480,000 cases are multidrug-resistant tuberculosis (MDR-TB),
15 accounting for 5% of all cases. Extensively drug-resistant (XDR) *Mycobacterium tuberculosis* and MDR-TB found in India are resistant to all anti-tuberculosis drugs.

 The current World Health Organization approved therapy for treating pulmonary non MDR-TB in adult patients is a four-drug combination of rifampicin, isoniazid, pyrazinamide, and ethambutol for six months. Although this treatment is very effective,
20 drug shortage and lack of effective supervision would affect the efficacy. This requires developing new drug or new drug combination therapy regimen to shorten the course of treatment. The treatment of MDR-TB cases requires administration of 2nd line drugs for at least 9 to 11 months. The 2nd line drugs include fluoroquinolones, amikacin, kanamycin, capreomycin and the like, which are toxic and can only cure 60% to 75%
25 cases when using the previously 13 to 26 months recommended W.H.O. regimen.

 Therefore, it is desirable to develop new anti-tuberculosis drugs and drug combination regimen to solve the current problems encountered in TB control.

SUMMARY OF INVENTION

30 It has been found that administering an ionophore and β -lactam antibiotic in combination may be used to treat tuberculosis. Surprisingly, this combination shows

synergistic potential, allowing the combination to be more effective than administering either antibiotic alone.

In one aspect, the present invention is to provide a pharmaceutical composition, comprising ionophore, β -lactam antibiotic and pharmaceutically acceptable carrier.

5 In a preferred embodiment, the ionophore is selected from the group consisting of nigericin, A23187, salinomycin, and the combination thereof.

In another preferred embodiment, the ionophore is nigericin.

In another preferred embodiment, the β -lactam antibiotic is selected from the group consisting of meropenem, amoxicillin, tebipenem, and the combination thereof.

10 In another embodiment, the β -lactam antibiotic is selected from the group consisting of meropenem, amoxicillin, and the combination thereof.

In another preferred embodiment, the β -lactam antibiotic is carbapenem antibiotic.

15 In another preferred embodiment, the pharmaceutical composition further comprises β -lactamase inhibitor.

In another preferred embodiment, the β -lactamase inhibitor is potassium clavulanate.

20 In another preferred embodiment, the weight ratio of the ionophore and β -lactam antibiotic is from 1:100 to 100:1, preferably 1:20 to 20:1, more preferably 1:5 to 5:1, most preferably 1:1.

In another preferred embodiment, the total content of the ionophore and β -lactam antibiotic is from 1 to 99%, preferably from 5 to 90% by weight of the pharmaceutical composition.

25 In another preferred embodiment, the weight ratio of ionophore, β -lactam antibiotic and β -lactamase inhibitor is from 1:100:1000 to 100:1:10, preferably from 1:20:500 to 20:1:25, more preferably is 4:1:250.

In another preferred embodiment, the total content of the ionophore, β -lactam antibiotic and β -lactamase inhibitor is from 1 to 99%, preferably from 5 to 90% by weight of the pharmaceutical composition.

30 In another preferred embodiment, the pharmaceutical composition is for the treatment of tuberculosis and/or for the inhibition of *Mycobacterium tuberculosis* complex (MTBC) strains multiplication.

In another preferred embodiment, the pharmaceutical composition is in an oral dosage form or in an injectable dosage form.

In another preferred embodiment, the pharmaceutical composition further comprises other pharmaceutically active ingredient, including active ingredient for the treatment of tuberculosis.

Another aspect of the present invention is to provide a use of the above pharmaceutical composition in the manufacture of a medicament for the treatment of tuberculosis and/or for inhibition of MTBC strains multiplication in a subject in need thereof.

In a preferred embodiment, the *Mycobacterium tuberculosis* is selected from the group consisting of drug-resistant *Mycobacterium tuberculosis*, multidrug-resistant *Mycobacterium tuberculosis*, extensively drug-resistant *Mycobacterium tuberculosis* and the combination thereof.

In a preferred embodiment, the inhibition of MTBC strains multiplication includes inhibition of the growth and reproduction of MTBC strains.

Another aspect of the present invention is to provide a kit comprising:

(i) a first compartment containing ionophore or a medicament comprising the ionophore;

(ii) a second compartment containing β -lactam antibiotic or a medicament comprising β -lactam antibiotic; and

(iii) instructions for treating tuberculosis and/or for inhibiting MTBC strains multiplication by the combination of ionophore and β -lactam antibiotic.

In a preferred embodiment, the kit further comprising:

(iv) a third compartment containing β -lactamase inhibitor or a medicament comprising the β -lactamase inhibitor.

In another preferred example, the instruction is further for treating tuberculosis and/or for inhibiting MTBC strains multiplication by the combination of ionophore, β -lactam antibiotic and β -lactamase inhibitor.

In another preferred embodiment, the medicaments in the first, the second and the third compartments are formulations containing the ionophore, β -lactam antibiotic and β -lactamase inhibitor, respectively.

In another preferred embodiment, the medicaments are in an oral dosage form or in an injectable dosage form.

In another preferred embodiment, the kit is for treatment of tuberculosis and/or for inhibition of MTBC strains multiplication in a subject in need thereof.

5 In another preferred embodiment, the ionophore, β -lactam antibiotic and β -lactamase inhibitor are administered simultaneously or sequentially.

Another aspect of the present invention is to provide a non-therapeutic method for inhibiting MTBC strains multiplication in vitro, comprising the step of incubating MTBC strains in the presence of ionophore, β -lactam antibiotics and optionally
10 β -lactamase inhibitors, to inhibit MTBC strains multiplication.

In a preferred embodiment, the ionophore is administrated at dose of 0.1 to 1 $\mu\text{g/ml}$, preferably 0.15 $\mu\text{g/ml}$.

In a preferred embodiment, the β -lactam antibiotic is administrated at dose of 0.01 to 0.1 $\mu\text{g/ml}$, preferably 0.018 $\mu\text{g/ml}$.

15 In a preferred embodiment, β -lactamase inhibitor is administrated at dose of 1 to 10 $\mu\text{g/ml}$, preferably 2.5 $\mu\text{g/ml}$.

Another aspect of the present invention is to provide a method for the treatment of tuberculosis and/or for the inhibition of MTBC strains multiplication, comprising the step of:

20 (i) administrating the following active ingredients to a subject in need thereof: (a) ionophore; (b) β -lactam antibiotic; and optionally (c) β -lactamase inhibitor.

In a preferred embodiment, the active ingredients are administrated simultaneously or sequentially.

25 In another preferred embodiment, said subject includes both human and non-human mammals.

In the aspects and embodiments as defined above, preferably, the MTBC strain is *Mycobacterium tuberculosis*.

It is to be understood that within the scope of the present invention, the above-described technical features of the present invention and the technical features
30 specifically described in the following (e.g., examples) may be combined with each other to form a new or preferred technical solution.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the changes in colony forming units (CFU) per milliliter of culture in presence of different drug or drug combination. Compared with individual administration, combined administration of nigericin and meropenem significantly inhibits the growth of BCG which belongs to the group of MTBC strains.

Figure 2 shows the synergistic interactions between A23187 and tebipenem (with 2.5 mg/L clavulanic acid) against intracellular MTBC strains. Drug concentrations are in mg/L.

DETAILED DESCRIPTION OF THE INVENTION

It has been surprisingly found by the inventors that, combination of ionophore and β -lactam antibiotics is very effective in inhibiting MTBC strains multiplication and exhibits significant synergistic effect. Experiments show that in MTBC strains cultures, compared to the individual administration with the same doses, the combination of ionophore and β -lactam antibiotics significantly decreased the colony formation units (CFU) in the cultures, showing synergistic effect. The present invention has been completed on the basis of this.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present invention, representative illustrative methods and materials are now described.

It is noted that, as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural referents unless the context clearly dictates otherwise. It is further noted that the claims may be drafted to exclude any optional element.

The words "comprises", "comprising", "include", "including", and "includes" when used in this specification and in the claims are intended to specify the presence of stated features, integers, components, or steps, but they do not preclude the presence or addition of one or more other features, integers, components, steps, or groups thereof.

The phrase "pharmaceutically acceptable" indicates that the substance or composition is compatible chemically and/or toxicologically, with the other ingredients comprising a formulation, and/or the mammal being treated therewith.

The terms "treat" or "treatment" refer to therapeutic, prophylactic, palliative or preventive measures. For purposes of this invention, beneficial or desired clinical results include, but are not limited to, alleviation of symptoms, diminishment of extent of disease, stabilized (i.e., not worsening) state of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable. Those in need of the treatment include those already with the condition or disorder, as well as those prone to have the condition or disorder or those for which the condition or disorder is to be prevented.

Tuberculosis is a chronic infectious disease caused by MTBC strains infection. MTBC strains may invade various organs of human body, but the main target is the lung, which is known as pulmonary tuberculosis.

The MTBC strains are a genetically related group of *Mycobacterium* species that can cause tuberculosis in humans or other living organisms. The MTBC strains include *Mycobacterium tuberculosis*, *Mycobacterium africanum*, *Mycobacterium orygis*, *Mycobacterium bovis* and the *Mycobacterium bovis* BCG strains, *Mycobacterium microti*, *Mycobacterium canetti*, *Mycobacterium caprae*, *Mycobacterium pinnipedii*, *Mycobacterium suricattae*, *Mycobacterium mungi*.

Multi-drug-resistant tuberculosis (MDR-TB) is a form of tuberculosis (TB) infection caused by MTBC strains that are resistant to at least two of the most powerful first-line anti-TB medications, isoniazid and rifampin.

Extensively drug-resistant tuberculosis (XDR-TB) is defined as TB due to MTBC strains that have developed resistance to at least rifampicin and isoniazid, as well as to any member of the quinolone family and at least one of the following second-line anti-TB injectable drugs: kanamycin, capreomycin, or amikacin.

As used herein, the terms "inhibition of MTBC strains multiplication" and "treatment of tuberculosis" are used interchangeably, including inhibition of the growth and reproduction of MTBC strains and extension of the tuberculosis disease.

Ionophore

Ionophore, also known as carboxyl polyethers, polyether antibiotics or polyether ionophore, forms electrically neutral complexes with monovalent or divalent cations, catalysing electrically silent exchanges of cations or protons across a variety of

biological membranes. These molecules function by rendering cell or intracellular membranes permeable to cations which are normally asymmetrically distributed across biological membranes thereby forming steep concentration gradients.

Example of Ionophore includes monensin (also known as A-3823A), narasin A
 5 (also known as A-28086A), narasin B(also known as A-28086B), narasin D (also known as A-28086D), lasalocid, salinomycin, and maduramicin, alborixin (also known as S-14750A, CP-38,986), laidlomycin (also known as AB-78), lenoremycin (also known as A-130A, Ro21-6150), A-130B, A-130C, dianemycin (also known as A-150, M5-16183), A-204A, A-204B, Ionomycin (also known as A-218), deoxylaidlomycin
 10 (also known as A-712), calcimycin (also known as A-23187), septamycin (also known as BL-580 α and A-28695A), A-28695B, K-41A (also known as A-32887), septamycin (also known as BL-580ab), BL-580 β , BL-580 δ , BL-580Z, carriomycin, cationomycin, chloronoboritomycin A (also known as X-14766A), etheromycin (also known as CP-38,295, C 20-12, T-40517), deoxy-salinomycin (also known as SY-1),
 15 deoxy-epi-salinomycin (SY-2), deoxy-narasin, deoxy-epi-narasin, dianemycon (also known as M5-16183, A-150), emeracid (also known as Ionomycin A and DE 3938), duamycin (also known as nigericin, helixin C, and azalomycin M), gridorixin, ionomycin, K-41B, lasalocid A (X-537A), lasalocid B, lasalocid C, lasalocid D, lasalocid E, iso-lasalocid A, leuseramycin, lomomycin B, lomomycin C, lysocellin,
 20 M-139603, monensin B, monensin C, monensin D, mutalomycin, noboritomycin A, noboritomycin B, RP 30504, RP 37454, salinomycin, salinomycin All, SY-4, SY-5, SY-8, tetronomycin, TM-531B, TM-531C, X-206, X-14547A, X-14667A, X-14667B, X-14868A, X-14868B, X-14868C, X-14868D, 5057, 6016.

Preferably the Ionophore is selected from the group comprising salinomycin;
 25 lasalocid; narasin; maduramicin; monensin, laidlomycin, and semduramicin. More preferably, the ionophore is selected from the group consisting of nigericin, A23187, salinomycin. More preferably, the ionophore is nigericin and/or A23187.

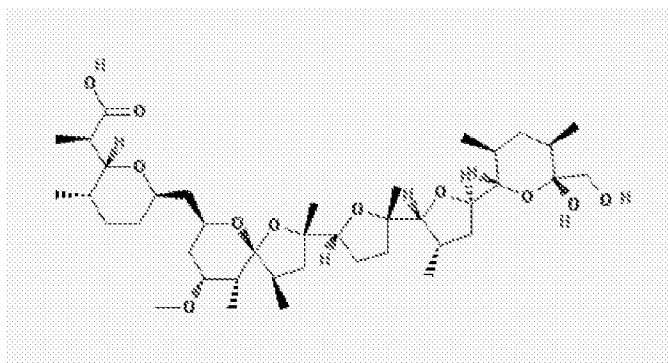
Nigericin

30 Nigericin is a polycyclic ether carboxylic acid compound. It mainly functions for the exchange between proton and potassium cation. Similar to the formation of complexes between valinomycin and potassium cation, the negatively charged

carboxylated nigericin interacts with the cation to form a nigericin-potassium complex for proton-potassium exchange.

Specifically, nigericin may form complexes with metal cations and penetrate the lipid bilayer and the cell membrane of the bacterium, thus changing the ion gradient in and outside the bacteria, increasing penetration into the bacterium, leading to bacterial swelling and forming vacuoles until cell death. At present, there has been no report on the role of nigericin in anti-mycobacterial infections.

The chemical formula of nigericin is $C_{40}H_{68}O_{11}$, with the molecular weight of 724.96. Its structure is shown as follows:



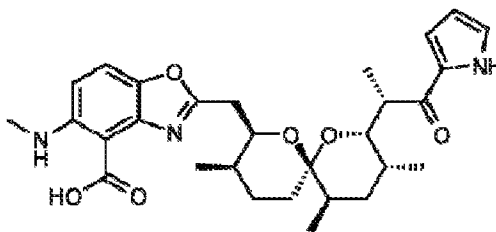
10

A23187

A23187 is a mobile ion-carrier that forms stable complexes with divalent cations. A23187 is also known as Calcimycin, Calcium Ionophore, Antibiotic A23187 and Calcium Ionophore A23187. It is produced at fermentation of *Streptomyces chartreusensis*.

A23187 has antibiotic properties against Gram-positive bacteria and fungi. It also acts as a divalent cation ionophore, allowing these ions to cross cell membranes, which are usually impermeable to them. It is used in laboratories to increase intracellular Ca^{2+} levels in intact cells. It also uncouples oxidative phosphorylation, the process cells use to synthesize Adenosine triphosphate for energy. In addition, A23187 inhibits mitochondrial ATPase activity. A23187 also induces apoptosis in some cells (e.g. mouse lymphoma cell line, or S49, and Jurkat cells) and prevents it in others (e.g. cells dependent on interleukin 3 that have had the factor withdrawn).

The chemical formula of A23187 is $C_{29}H_{37}N_3O_6$, with the molecular weight of 523.62. Its structure is shown as follows:



β -lactam antibiotic

β -lactam antibiotic (β -lactam) refer to a large class of antibiotics with the β -lactam ring in their chemical structures and is therefore susceptible to degradation by β -lactamases. Such antibiotics have the advantages of strong bactericidal activity, low toxicity, applicability to broad indications and excellent clinical efficacy. The modification to the chemical structure, especially the side chain of such drugs forms a number of antibiotics with different antibacterial profile and antibacterial effect, as well as a variety of clinical pharmacological properties.

β -lactam is the most widely used antibiotic. It induces cell apoptosis mainly by inhibiting synthesis and metabolism of bacterial peptidoglycan. Historically, β -lactam antibiotics have not been used in the treatment of tuberculosis, except for those multidrug-resistant MTBC infection cases in which existing treatments do not work. The reason for this is mainly due to β -lactamase BlaC encoded by the MTBC chromosome, as well as low permeability of such drugs in the bacterial cell wall.

Non-limiting examples of β -lactam antibiotic include the most commonly used penicillin and cephalosporins, as well as the recently developed cephamecin, thiomycin, monocyclic β -lactam and other atypical β -lactam antibiotics.

Non-limiting examples of β -lactam antibiotic useful with respect to the invention include penicillins, cephalosporins, penems, carbapenems, and monobactams.

Non-limiting examples include carbapenems (e.g. meropenem, faropenem, imipenem, ertapenem, doripenem, panipenem/betamipron and biapenem as well as razupenem, tebipenem, lenapenem and tomopenem), ureidopenicillins (e.g. piperacillin), carbacephems (e.g. loracarbef) and cephalosporins (e.g. cefpodoxime, ceftazidime, cefotaxime, ceftriaxone, ceftobiprole, and ceftaroline). Specific examples of β -lactam antibiotic agents include temocillin, piperacillin, cefpodoxime, ceftazidime, cefotaxime, ceftriaxone, meropenem, faropenem, imipenem, loracarbef, ceftobiprole, ceftaroline.

Carbapenem antibiotics are atypical β -lactam antibiotics with the widest antibacterial profile and the strongest antibacterial activity. Their structures are similar with the penicillium ring of penicillin, except for the sulfur atom on the thiazolidine ring replaced by carbon, and the unsaturated double bond between C2 and C3. In addition,
5 its 6-hydroxyethyl side chain is in a trans-conformation. Their affinity with BlaC is very low and is not affected by the β -lactamase activity.

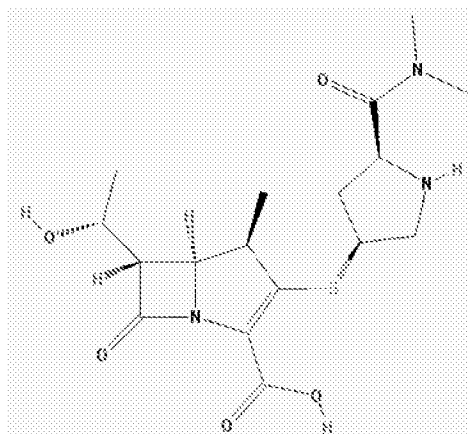
In the art, combination of β -lactam antibiotics and potassium clavulanate is conventionally used clinically.

In the present invention, the preferred β -lactam antibiotics include meropenem,
10 amoxicillin and tebipenem.

Meropenem

As used herein, meropenem, a β -lactam antibiotic classified as carbapenem, is an injectable antibiotic for the treatment of a variety of different infections, with very
15 broad antibacterial profile.

The chemical formula of meropenem is $C_{17}H_{25}N_3O_5S$, with the molecular weight of 383.46. Its structure is shown as follows:

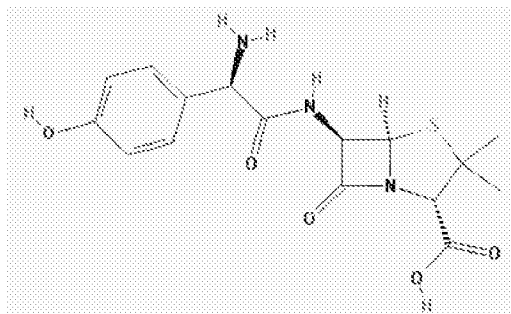


20 Amoxicillin

Amoxicillin is one of the most commonly used semi-synthetic penicillin-based broad-spectrum β -lactam antibiotics. It is present as white powder with a half-life of about 61.3 minutes. It is stable under acidic conditions, with 90% absorbed in gastrointestinal tract. Amoxicillin has a strong anti-bacterial activity, as well as strong
25 ability to penetrate the cell membrane. It is one of the most widely used semi-synthetic

oral penicillin, prepared as capsules, tablets, granules, dispersible tablets, and the like, and now often forms dispersible tablets with clavulanic acid.

The chemical formula of Amoxicillin is $C_{16}H_{19}N_3O_5S$, with the molecular weight of 365.411. Its structure is shown as follows:



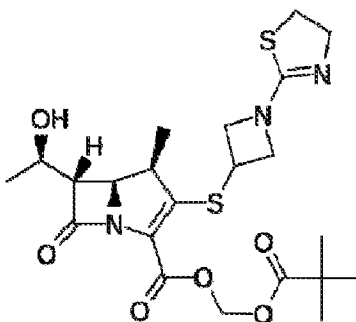
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Tebipenem

Tebipenem is a broad-spectrum orally-administered antibiotic, from the carbapenem subgroup of β -lactam antibiotics. It binds to bacterial penicillin-binding protein (PBP), inhibits the synthesis of bacterial cell walls, and is an oral carbapenem antibiotic.

10

The chemical formula of tebipenem is $C_{22}H_{31}N_3O_6S_2$, with the molecular weight of 497.63. Its structure is shown as follows:



15

β -lactamase inhibitor

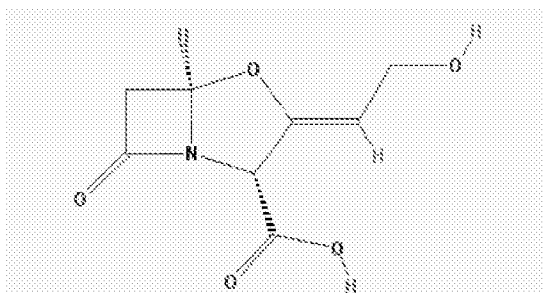
The agent able to inhibit beta-lactamase includes Clavulanic Acid, Sulbactam, Sultamicillin, Tazobactam, Renal Dipeptidase Inhibitors (Cilastatin), and Renal Protectant (Betamipron).

20

Clavulanic acid

Clavulanic acid is an irreversible competitive β -lactamase inhibitor. It causes enzyme inactivation after binding with the enzyme, and thus exhibits a strong inhibitory activity. Clavulanic acid not only acts on *Staphylococcus aureus* β -lactamase, but also inhibits Gram-negative bacillus β -lactamase. Its combination with penicillins and/or
5 cephalosporins significantly increases the antibacterial activity of beta-lactams and decreases their minimum inhibitory concentration (MIC). As a result, the drug efficacy is increased up to more than ten times, causing the resistant strains to recover its sensitivity.

The chemical formula of clavulanic acid is $C_8H_9NO_5$, with the molecular weight
10 of 199.16. Its structure is shown as follows:



In the combination, composition, use, method and kit of the present invention, the ionophore may be administered prior to, simultaneously with, or subsequent to a β -lactam antibiotic (“co-administration”). The ionophore and β -lactam antibiotic may be
15 administered separately by different routes, if desired. As used herein, the term administration or administering is used to denote simultaneous or sequential administration. Preferably, such administration produces a synergistic effect.

As used herein, the term “administering” refers to bringing a subject, tissue, organ or cells in contact with the combination or composition described in this
20 disclosure. In certain embodiments, the present invention encompasses administering the combination or composition described in this disclosure to a patient or subject.

A “subject,” “patient” and “individual,” used equivalently herein, refers to a mammal, preferably a human, that either: (1) is developing tuberculosis due to MTBC strains, such disease being remediable, treatable, or diminished in severity by
25 administration of the combination or composition according to the invention; or (2) is susceptible to such an infection that is preventable by administering same.

The terms “synergy” and “synergistic effect” indicate that the effect produced when two or more drugs are co-administered is greater than would be predicted based on the effect produced when the compounds are administered individually. In general, a synergistic effect is most clearly demonstrated at sub-optimal concentrations of the compounds (i.e., sub-therapeutic dosages). A lower dosage minimizes the potential of side effects, thereby providing an increased margin of safety. Synergy can result in lower cytotoxicity, increased anti-tuberculosis effect, or some other beneficial effect of the combination compared with the individual components.

One embodiment provides a combination, comprising: (a) an ionophore; and (b) a β -lactam antibiotic. In a further embodiment, the ionophore is selected from the group as defined above. In a further embodiment, the ionophore is selected from the group consisting of nigericin, A23187, salinomycin, and the combination thereof. In a further embodiment, the β -lactam antibiotic is selected from the group as defined above. In a further embodiment, the β -lactam antibiotic is carbapenem or penicillins antibiotic. In a further embodiment, the β -lactam antibiotic is selected from the group consisting of meropenem, amoxicillin and tebipenem. In a further embodiment, the weight ratio of the ionophore and the β -lactam antibiotic is from 1:100 to 100:1, preferably 1:20 to 20:1, more preferably 1:5 to 5:1, more preferably 1:1.

In a further embodiment, the combination further comprises (c) β -lactamase inhibitor. In a further embodiment, the β -lactamase inhibitor is selected from the group as defined above. In a further embodiment, the β -lactamase inhibitor is potassium clavulanate. In a further embodiment, the weight ratio of the ionophore, the β -lactam antibiotic and the β -lactamase inhibitor is from 1:100:1000 to 100:1:10, more preferably 1:20:500 to 20:1:25, most preferably 4:1:250.

In a further embodiment, it provides a combination, comprising: (a) an ionophore is selected from the group consisting of nigericin, A23187, salinomycin; and (b) a β -lactam antibiotic selected from the group of carbapenem or penicillins antibiotic. In a further embodiment, the weight ratio of the ionophore to the β -lactam antibiotic is from 1:100 to 100:1, preferably 1:20 to 20:1, more preferably 1:5 to 5:1, more preferably 1:1.

In a further embodiment, it provides a combination, comprising: (a) an ionophore is selected from the group consisting of nigericin, A23187, salinomycin; and (b) a β -lactam antibiotic selected from the group of meropenem, amoxicillin and tebipenem. In a further embodiment, the weight ratio of the ionophore to the β -lactam antibiotic is from 1:100 to 100:1, preferably 1:20 to 20:1, more preferably 1:5 to 5:1, more preferably 1:1.

In a further embodiment, it provides a combination, comprising A23187 and tebipenem. In a further embodiment, the weight ratio of the ionophore to the β -lactam antibiotic is from 1:100 to 100:1, preferably 1:20 to 20:1, more preferably 1:5 to 5:1, more preferably 1:1.

In a further embodiment, it provides a combination, comprising nigericin and meropenem. In a further embodiment, the weight ratio of the ionophore to the β -lactam antibiotic is from 1:100 to 100:1, preferably 1:20 to 20:1, more preferably 1:5 to 5:1, more preferably 1:1.

In a further embodiment, it provides a combination, comprising nigericin, amoxicillin and potassium clavulanate. In a further embodiment, the weight ratio of the ionophore, the β -lactam antibiotic and the β -lactamase inhibitor is from 1:100:1000 to 100:1:10, more preferably 1:20:500 to 20:1:25, most preferably 4:1:250.

Another embodiment provides a pharmaceutical composition comprising: (a) an ionophore; (b) a β -lactam antibiotic; and optionally (c) a pharmaceutically acceptable carrier. In a further embodiment, the ionophore is selected from the group as defined above. In a further embodiment, the ionophore is selected from the group consisting of nigericin, A23187, salinomycin, and the combination thereof. In a further embodiment, the β -lactam antibiotic is selected from the group as defined above. In a further embodiment, the β -lactam antibiotic is carbapenem or penicillins antibiotic. In a further embodiment, the β -lactam antibiotic is selected from the group consisting of meropenem, amoxicillin, and tebipenem. In a further embodiment, the weight ratio of the ionophore and the β -lactam antibiotic is from 1:100 to 100:1, preferably 1:20 to 20:1, more preferably 1:5 to 5:1, most preferably 1:1.

In a further embodiment, the composition further comprises β -lactamase inhibitor. In a further embodiment, the β -lactamase inhibitor is selected from the group

as defined above. In a further embodiment, the β -lactamase inhibitor is potassium clavulanate. In a further embodiment, the weight ratio of the ionophore, the β -lactam antibiotic and the β -lactamase inhibitor is from 1:100:1000 to 100:1:10, preferably 1:20:500 to 20:1:25, more preferably 4:1:250.

5 In a further embodiment, it provides a composition, comprising: (a) an ionophore selected from the group consisting of nigericin, A23187, salinomycin; and (b) a β -lactam antibiotic selected from the group of carbapenem or penicillins antibiotic. In a further embodiment, the weight ratio of the ionophore to the β -lactam antibiotic is from 1:100 to 100:1, preferably 1:20 to 20:1, more preferably 1:5 to 5:1, more
10 preferably 1:1.

 In a further embodiment, it provides a composition, comprising: (a) an ionophore is selected from the group consisting of nigericin, A23187, salinomycin; and (b) a β -lactam antibiotic selected from the group of meropenem, amoxicillin and tebipenem. In a further embodiment, the weight ratio of the ionophore to the β -lactam
15 antibiotic is from 1:100 to 100:1, preferably 1:20 to 20:1, more preferably 1:5 to 5:1, more preferably 1:1.

 In a further embodiment, it provides a composition, comprising A23187, and tebipenem. In a further embodiment, the weight ratio of the ionophore to the β -lactam antibiotic is from 1:100 to 100:1, preferably 1:20 to 20:1, more preferably 1:5 to 5:1,
20 more preferably 1:1.

 In a further embodiment, it provides a composition, comprising nigericin and meropenem. In a further embodiment, the weight ratio of the ionophore to the β -lactam antibiotic is from 1:100 to 100:1, preferably 1:20 to 20:1, more preferably 1:5 to 5:1, more preferably 1:1.

25 In a further embodiment, it provides a composition, comprising nigericin, amoxicillin and potassium clavulanate. In a further embodiment, the weight ratio of the ionophore, the β -lactam antibiotic and the β -lactamase inhibitor is from 1:100:1000 to 100:1:10, more preferably 1:20:500 to 20:1:25, most preferably 4:1:250.

 Another embodiment provides use of the combination defined as above or the
30 composition defined as above in the manufacture of medicament for treatment of tuberculosis or for inhibiting MTBC strains multiplication. In a further embodiment, the MTBC strains are selected from the group consisting of drug-resistant MTBC strains,

multidrug-resistant MTBC strains, extensively drug-resistant MTBC strains and the combination thereof. In a further embodiment, the ionophore, β -lactam antibiotic and optionally β -lactamase inhibitor are provided simultaneously, sequentially or separately.

Another embodiment provides a method for treating tuberculosis or for
5 inhibiting *Mycobacterium tuberculosis* complex strains multiplication, comprising administering the combination as defined above or the composition as defined above to a subject in need thereof. In a further embodiment, the MTBC strains are selected from the group consisting of drug-resistant MTBC strains, multidrug-resistant MTBC strains, extensively drug-resistant MTBC strains and the combination thereof.

10 Another embodiment provides a kit, comprising:

i) a first compartment containing ionophore or a medicament comprising the ionophore;

ii) a second compartment containing β -lactam antibiotic or a medicament comprising the β -lactam antibiotic; and

15 iii) instructions for use in treating tuberculosis and/or for inhibiting MTBC strains multiplication.

In a further embodiment, the kit as defined above, further comprising:

iv) a third compartment containing β -lactamase inhibitor or a medicament comprising the β -lactamase inhibitor.

20 In the embodiments as defined above, preferably, the MTBC strain is *Mycobacterium tuberculosis*.

Pharmaceutical composition and kit

The present invention provides pharmaceutical composition comprising: (a)
25 ionophore; (b) β -lactam antibiotics; optionally (c) β -lactamase inhibitors; and (d) pharmaceutically acceptable carriers. Said carriers include but are not limited to: saline, buffers, glucose, water, glycerol, ethanol, powders, and combinations thereof. The pharmaceutical formulation matches with the administration route. The pharmaceutical compositions of the present invention may be prepared as injections, for example,
30 prepared through conventional methods using physiological saline or aqueous solutions containing glucose and other adjuvants. Pharmaceutical compositions such as tablets and capsules can be prepared by conventional methods. Pharmaceutical compositions

such as injections, solutions, tablets and capsules should be prepared under aseptic conditions. The pharmaceutical combination of the present invention may also be formulated as a powder for inhalation. One preferred dosage form is oral formulation. In addition, the pharmaceutical compositions of the present invention may also be administered with other therapeutic agents.

The present invention further provides a kit for treatment of MTBC strains multiplication, comprising:

- (i) a first compartment containing ionophore or a medicament comprising the ionophore;
- 10 (ii) a second compartment containing β -lactam antibiotic or a medicament comprising β -lactam antibiotic; and
- (iii) optionally a third compartment containing β -lactamase inhibitor or a medicament comprising β -lactamase inhibitor; and
- 15 (iv) instructions for inhibition of MTBC strains multiplication by administering ionophore, β -lactam antibiotic and β -lactamase inhibitor in combination.

In the pharmaceutical composition or the kit of the present invention, the weight ratio of ionophore, β -lactam antibiotic and β -lactamase inhibitor is from 1:100:1000 to 100:1:10, preferably from 1:20:500 to 20:1:25, most preferably 4:1:250.

The pharmaceutical composition and kit of the present invention is suitable for the treatment of tuberculosis, preferably for the treatment of drug-resistant tuberculosis, more preferably for the treatment of multidrug-resistant tuberculosis.

The pharmaceutical composition and kit of the present invention is suitable for the inhibition of MTBC strains multiplication. Said MTBC strains include drug-resistant MTBC strains, multidrug-resistant MTBC strains and extensively drug-resistant MTBC strains. Preferably, in the above compositions and kits of the present invention, the MTBC strain is *Mycobacterium tuberculosis*.

The formulations of the present invention may be administered three or four times per day, or administered once a day in a sustained release manner. Preferably, the formulation is administered once a day, thereby significantly improving patient compliance.

At the time of administration, the vast majority of patients generally have a daily dose of less than (or in a few cases equal to or slightly greater than) the daily dose of

each individual drug. The effective dose of the active ingredient may vary with the severity of the disease to be treated, and the like.

Therapeutic Method

5 The present invention also provides a method of treating tuberculosis using the three active ingredients of the invention or the corresponding medicament. Said method comprises the step of administering to human or non human living organisms an effective amount of: (a) an ionophore; (b) a β -lactam antibiotic; and optionally (c) a β -lactamase inhibitor, or comprises the step of administering a pharmaceutical
10 composition comprising (a), (b) and (c).

 When the active ingredients of the present invention are used for the above purposes, they may be mixed with one or more pharmaceutically acceptable carriers or excipients such as solvents, diluents and the like. They may be administered orally in the following forms: tablets, pills, capsules, dispersible powders, granules or
15 suspensions (containing, for example, about 0.05-5% suspending agent), syrups (containing, for example, about 10-50% sugar), and elixirs (containing about 20-50% ethanol). Alternatively, they may also be administered parenterally in the form of a sterile injectable solution or suspension (containing about 0.05-5% suspending agent in the isotonic medium). For example, such pharmaceutical formulations may contain
20 from about 0.01% to about 99%, more preferably from about 0.1% to about 90% by weight of the active ingredients, which are mixed with the carrier.

 The active ingredients or the pharmaceutical compositions of the present invention may be administered through conventional route, including, but not limited to, intramuscular, intraperitoneal, intravenous, subcutaneous, intradermal, oral, or topical
25 administration. Preferred administration routes include oral, intramuscular or intravenous administration.

 From the standpoint of ease of administration, preferred pharmaceutical compositions are solid compositions, particularly oral agents.

 The pharmaceutical compositions of the present invention are able to inhibit the
30 growth of MTBC strains, exhibits significant synergistic effect, and have the potential to be used in the treatment of multidrug-resistant tuberculosis.

The invention will now be further described with reference to specific embodiments. It is to be understood that these examples are given for illustration purpose only and are not intended to limit the scope of the invention. The experimental methods not specified for the specific conditions in the following examples are generally in accordance with conventional conditions or in accordance with the conditions recommended by the manufacturer. Unless otherwise stated, the percentages as described are by weight.

EXAMPLES

10 Materials and Methods

The formulations of the media involved in the Examples are as follows:

Formulation of 7H9 broth

Component	Amount
Ammonia Sulfate	0.5000 g
Potassium Phosphate Monobasic	1.0000 g
Sodium Phosphate Dibasic	2.5000 g
Sodium Citrate	0.1000 g
Magnesium Sulfate	0.0500 g
Calcium Chloride	0.0005 g
Zinc Sulfate	0.0010 g
Copper Sulfate	0.0010 g
L-Glutamic acid	0.5000 g
Ammonium Ferric Citrate	0.0400 g
Pyridoxol	0.0010 g
Biotin	0.0005 g
pH	6.6 ± 0.2

15 Formulation of ADC Enrichment Broth

Component	Amount
Fifth Component of Bovine Serum Albumin V	5.000 g
Glucose	2.000 g
Catalase	0.003 g

The above components are dissolved in 100 ml distilled water, and are filter sterilized through 0.2 µm membrane.

Formulation of 7H9 broth

20 4.7 g of 7H9 broth is dissolved in 900 ml deionized water with 2 ml glycerol and heated until complete dissolution. The solution is autoclaved at 121 °C and then cooled

to 50-55°C. Under aseptic conditions, 100 ml ADC is added into the solution and thoroughly mixed.

Formulation of the 7H11 broth

Component	Amount
Casein Tryptone	1.0000 g
L-Glutamic acid	0.5000 g
Sodium Citrate	0.4000 g
Pyridoxol	0.0010 g
Biotin	0.0005 g
Ammonium Ferric Citrate	0.0400 g
Ammonia Sulfate	0.5000 g
Sodium Phosphate Dibasic	1.5000 g
Potassium Phosphate Monobasic	1.5000 g
Magnesium Sulfate	0.0500 g
Agar	15.0 g
Malachite Green	0.0010 g
pH	6.6 ± 0.2

5

Formulation of OADC Enrichment Broth

Component	Amount
Bovine Serum Albumin V	5.000 g
Glucose	2.000 g
Catalase	0.003 g
Oleic Acid	0.06 ml

The above components are dissolved in 100 ml distilled water, and are filter sterilized through 0.2 µm membrane.

10 Formulation of 7H11 broth

21 g of 7H11 broth is dissolved in 900 ml deionized water with 5 ml glycerol and heated until complete dissolution. The solution is autoclaved at 121°C and then cooled to 50-55°C. Under aseptic conditions, 100 ml OADC is added into the solution and thoroughly mixed.

15

Time kill method

The time-kill method is to compare the amount of cultivable bacteria in the culture after each component is administered to the bacteria individually or in combination. The criteria for the evaluation are that, compared to the individual administration at the same concentration, the number of cultivable bacteria after

20

combined administration exhibits a 2 log₁₀ decrease, it is determined that, synergistic effect is achieved with the combined administration.

Chequerboard titration test

- 5 Chequerboard titration test method is to use the method of checkerboard titration in the microplate to determine the minimum inhibitory concentration of each component when administered in combination.

For a drug, the fractional inhibitory concentration (FIC) index was calculated using the following formula: FICA= (MIC of drug A in the presence of drug B)/ (MIC of drug A alone). For a combination of two drugs (namely A and B), the FICI index of the combination was determined by adding the two FIC index of the individual drugs (FICA+ FICB). Synergy, antagonism and no interaction were defined as FICI ≤ 0.5 , FICI > 4.0 and FICI = 0.5-4.0, respectively (Odds FC. Synergy, antagonism, and what the chequerboard puts between them. J Antimicrob Chemother. 2003; 52:1).

- 15 *M. marinum* (ATCC BAA-535) is a human patient isolate from Moffett Hospital, University of California, San Francisco in 1992. Multi-locus sequence typing shows that this strain belongs to sequence type 22 (Yip MJ et al. Evolution of *Mycobacterium ulcerans* and other mycolactone-producing mycobacteria from a common *Mycobacterium marinum* progenitor. J. Bacteriol. 2007; 189: 2021–2029).

- 20 *M. smegmatis* strain is the *M. smegmatis* mc2 155 strain described in “Isolation and characterization of efficient plasmid transformation mutants of *Mycobacterium smegmatis*” by Snapper SB, Melton RE, Mustafa S, Kieser T, Jacobs WR Jr. Mol Microbiol. 1990 Nov; 4(11): 1911-9.

- 25 *M. aurum* is the stain ATCC 23366 kept in the collection of Institut Pasteur under the number CIP104465T. It is described in Int. J. Syst. Bacteriol., 1980 30 : 325 and J. Gen. Microbiol., 1966 45 : 253.

- M. abscessus* ATCC 19977 type strain (Moore and Frerichs, 1953) was from the American Type Culture Collection (ATCC) and obtained from the collection of the Institut Pasteur (CIP), where the ATCC 19977T strain was previously regrown and listed as CIP104536.

M. avium is from the ATCC collection ATCC 25291.

M. tuberculosis H37Rv is from the ATCC collection (ATCC 27294).

Mycobacterium bovis BCG (BCG)

BCG is a vaccine for the prevention of tuberculosis and is prepared using viable *Mycobacterium bovis* BCG that are non-pathogenic for non immune-compromised living organisms.

5 BCG is a member of the group of MTBC strains, and the inventors have found the synergistic effects of ionophore and β -lactam antibiotics in BCG. This will help to further develop a new anti-bacterial drug combination. Such combination can also be used for the treatment of multidrug-resistant tuberculosis and to enhance the function of β -lactam in its existing antibacterial profile.

10 *M. bovis* BCG strain 1173 P2 is described in “The stability and immunogenicity of a dispersed-grown freeze-dried Pasteur BCG vaccine” by Gheorghiu M, Lagrange PH, Fillastre C. J Biol Stand. 1988 Jan; 16(1):15-26.

M. bovis BCG was cultured in 7H9 OADC media (Beckton Dickinson) and kept frozen at -20°C in tubes after addition of 50% glycerol (final concentration) for
15 conservation (the cryopreserved BCG).

Example 1

The synergistic effect of nigericin and meropenem studied by time-kill method

20 The cryopreserved BCG was thawed and inoculated into 7H9 broth, incubated at 37°C, 220 rpm until OD600 achieved 0.6-0.8.

The above cultures were inoculated at a ratio of 1: 100 into 7H9 broth containing 0.31 $\mu\text{g/ml}$ of nigericin or 0.31 $\mu\text{g/ml}$ of meropenem, respectively. It was also inoculated into a 7H9 broth containing 0.31 $\mu\text{g/ml}$ of nigericin and 0.31 $\mu\text{g/ml}$ of
25 meropenem in the same proportion. An antibiotic-free 7H9 broth was used as a control.

In the broth containing both nigericin and meropenem, said nigericin and meropenem were formulated in a weight ratio of 1:1 (i.e. molar ratio of 1:1.89).

From the time of inoculation, 10 μl of culture was taken and inoculated to the 7H11 solid plate at a ratio of 1: 10^5 every day, and the number of colonies was
30 calculated after 3-4 weeks of inverted incubation at 37 °C.

The results are shown in Figure 1 and Table 1. On the 6th day of incubation, compared to the medium containing nigericin or meropenem at the same dose, the

colony forming units (CFU) in the medium with both nigerincin and meropenem were reduced by 10^4 . The result shows the synergistic effect between nigerincin and meropenem, as the combined administration of these drugs reduces the BCG by 10^4 CFU in 6 days, compared to the individual administration. Such synergistic effect is achieved at half of the minimum inhibitory concentration of nigerincin and a quarter of the minimum inhibitory concentration of meropenem. All the experiments were repeated three times.

Table 1. Synergistic effect of nigerincin and meropenem determined by Time-kill method

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Control	5.0*	12.6	6.3	63.1	10.0	15.8	15.8
Nigerincin	5.0	6.3	1.0	0.8	1.6	3.2	3.2
Meropenem	5.0	12.6	1.3	1.0	2.5	6.3	6.3
Nigerincin + Meropenem	5.0	5.0	0.6	0.3	0.1	0.03	0.0003

*Colony Forming Units $\times 10^6$ (CFU/ml)

Example 2

Synergistic effect of nigerincin, amoxicillin and potassium clavulanate studied by Chequerboard titration test method

The following experiment was carried out using Chequerboard titration test method.

The cryopreserved BCG was thawed and inoculated into 7H9 broth. The culture was incubated at 37°C, 220 rpm until OD600 = 0.6-0.8. Then the OD600 of the culture was adjusted to 0.2-0.4 with the 7H9 broth.

In the 96-wells plate, the nigerincin was serially diluted from column to column, and the concentration was from 50 μ g/ml to 0.39 μ g/ml. The amoxicillin was serially diluted from row to row, and the concentration is from 2.5 μ g/ml to 0.009 μ g/ml. To each well, the potassium clavulanate was added until a final concentration of 2.5 μ g/ml. The final volume for each well was 100 μ l.

For each well, 10 μ l culture with OD600 being 0.2-0.4 was added.

The samples were added in an 8x8 manner.

According to the result, the anti-BCG FICI value of the above combination (nigericin + amoxicillin + potassium clavulanate) was 0.5. According to Chequerboard titration test method, $FICI \leq 0.5$ is a criteria of the synergistic effect between a combination. In this regard, the combination of nigericin, amoxicillin and potassium clavulanate brought synergistic effect.

Under the following condition, the anti-bacteria effect was maximized and the synergistic effect is significant: nigericin: 0.15 $\mu\text{g/ml}$; amoxicillin: 0.018 $\mu\text{g/ml}$; and potassium clavulanate: 2.5 $\mu\text{g/ml}$ (i.e. the molar concentration ratio of nigericin : amoxicillin : potassium clavulanate is 4:1:250).

Example 3

Drug and reagents preparation

Antibiotic solutions were prepared at a concentration of 1 mg/ml in distilled water, filter sterilized, and frozen until use. All compounds to be screened were dissolved in 100% dimethylsulfoxide (DMSO) and stored as frozen stocks at a concentration of 2 mg/ml. Resazurin sodium powder was prepared at 0.01% (w/v) in distilled water, filter sterilized, and stored at 4°C for up to one week. All chemicals were obtained from Sigma-Aldrich, USA.

Strains and growth conditions

M. marinum, *M. abscessus*, *M. avium*, and *M. bovis* BCG and *M. tuberculosis* H37Rv were cultured in Middlebrook 7H9 broth containing 10% (v/v) ADC enrichment (albumin, dextrose, catalase; Becton Dickinson) and 0.05% Tween 80, or Middlebrook 7H11 agar medium containing 10% (v/v) OADC enrichment (oleic acid, albumin, dextrose, catalase; Becton Dickinson). *M. aurum* and *M. smegmatis* were grown in LB broth containing 0.05% Tween 80, or LB agar containing Difco agar (1.5%). All mycobacteria were cultured at 37°C except for *M. marinum* that was cultured at 30°C.

High-throughput screening, MIC determination

The compounds tested during this study were purchased from Topscience Co., Ltd, China. They were screened in search of their activity against whole-cells of *M. aurum* as determined using the resazurin-reduction assay. A fresh culture of *M. aurum*

in exponential phase in LB broth ($OD_{600} \sim 0.6-0.8$) was diluted to $OD_{600} \sim 0.2-0.3$ in the same culture medium. A mixture of 50 μ L fresh LB broth, 2.5 μ L of the diluted *M. aurum* suspension, and 15 μ L resazurin solution was added to each of 384-well plates (Thermo Scientific). The *M. aurum* inoculum was replaced by 2.5 μ L LB medium as a sterility control. Control antibiotics and the compounds were tested at a final concentration of 10 mg/L. The plates were incubated 18 h at 37°C. The read-out was based on cell viability as measured by the resazurin-reduction microplate assay (Palomino JC, Martin A, Camacho Met al. Resazurin microtiter assay plate: simple and inexpensive method for detection of drug resistance in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 2002; **46**: 2720-2).

Primary hits were filtered first by an activity cut-off (60% inhibition) as well as by selecting for drug-like properties. Secondly, chemical clustering further reduced the number of compounds that were then used to evaluate their efficacy by determining their MIC using the resazurin susceptibility assay as already described. For this, serial two-fold dilutions of each compound were prepared directly in a sterile 96-well microtiter plate (Thermo Fisher) using 100 μ L LB broth or Middlebrook 7H9 broth medium, depending on the mycobacterial species being used. A fresh culture of *M. aurum* in exponential phase in LB broth ($OD_{600} \sim 0.6-0.8$) was diluted to $OD_{600} \sim 0.2-0.3$ in the same culture medium. A mixture of 5 μ L of this bacterial suspension and 30 μ L resazurin solution was added to each well. The plates were incubated 18h at 37°C. For assays with *M. marinum*, BCG and *M. tuberculosis*, we added 5 μ L of a diluted culture ($OD_{600} \sim 0.2-0.3$) and then added 30 μ L resazurin on the third and seventh day of incubation and read the plate 18h later. Change in color from blue to pink indicated bacterial growth. The MICs were defined as the lowest concentration of compound that prevented this change in color.

Interaction between ionophore and other anti-mycobacterial drug in vitro

Drug interactions between the ionophore and other anti-mycobacterial drugs (rifampicin, ofloxacin, amikacin, kanamycin, beta-lactam with or without clavulanic acid, nigericin and A23187, at a range of concentrations between 5 and 0.039 mg/L) were performed with *M. tuberculosis* cultures, using resazurin as a viability marker in the chequerboard titration assay (Chen P, Gearhart J, Protopopova Met al. Synergistic

interactions of SQ109, a new ethylene diamine, with front-line antitubercular drugs in vitro. *J Antimicrob Chemother* 2006; **58**:332-7.).

Interaction between A23187 and Tebipenem with clavulanate against *M. tuberculosis* in infected macrophage

Macrophages were infected *M. tuberculosis* H37Rv at a multiplicity of infection of 0.5 bacteria/cell. The infected cells were washed and incubated in fresh medium with or without drugs after 2 hours of infection. After an additional 6 days culture, cells were lysed by resuspension in 0.1% Triton X-100 in distilled water and bacteria enumeration were done after 3 weeks at 37°C (Tailleux L, Neyrolles O, Honore-Bouakline S, et al. Constrained intracellular survival of *Mycobacterium tuberculosis* in human dendritic cells, *J Immunol*, 2003, vol. 170: 1939-48). We used 0.08 and 0.16 mg/L A23187 to combine the concentrations of 0.625 and 1.25 mg/L tebipenem with clavulanate (2.5 mg/L).

Statistical methods

Data are presented as the mean $\pm$ SD and significance was calculated using the independent T-test using SPSS software version 17.0. Results were considered to be statistically significant when $P < 0.05$.

Results

Screening of chemical library

Several compounds in the library were already known for their antibacterial activity, such as the rifamycins and neomycin, thus showing the efficacy of our screening system for the isolation of molecules with anti-bacterial activity. The activities of these compounds were evaluated by determining their MICs for several *Mycobacterium* species: *M. aurum*, *M. smegmatis*, *M. marinum*, and *M. bovis* BCG. The results of the MIC determinations are shown in Table 2. Three compounds (nigericin, salinomycin, and A23187) had lower MICs in *M. aurum* (0.16, 0.08, and 0.04 mg/L respectively) than in other mycobacterial species, and they were selected for further studies. The MIC of A23187 in *M. smegmatis* was 0.31 mg/L and that of nigericin in BCG was 0.625 mg/L. These three compounds are ionophores, known to promote ion

transport through cell membranes.

The MICs of these three ionophores were also determined in *M. Tuberculosis* and two other pathogenic mycobacterial species, *M. abscessus* and *M. avium*., *M. tuberculosis* was fully susceptible to nigericin and A23187 whereas *M. abscessus* and *M. avium* appeared to be naturally resistant to all three compounds. Salinomycin showed higher MIC with *M. tuberculosis* and was not retained for further studies.

Out of all mycobacterial species tested, *M. aurum* was the most sensitive to the three ionophores.

Table 2. *In vitro* anti-mycobacterial activity of ionophores

Strain	MICs (mg/L)		
	Nigericin	A23187	Salinomycin
<i>M. aurum</i>	0.16	0.08	0.04
<i>M. smegmatis</i>	2.5	0.31	3.125
<i>M. marinum</i>	2.5	5	10
<i>M. abscessus</i>	>20	>20	>20
<i>M. avium</i>	>20	12.5	10
<i>M. bovis</i> BCG	0.625	1.25	6.25
<i>M. tuberculosis</i>	1.25	1.25	10

Synergistic interaction between ionophore and beta-lactam antibiotic in vitro

The interactions between ionophore and other anti-mycobacterial drug were studied by the chequerboard method. There were two combinations (nigericin and A23187 with the combination of tebipenem/clavulanic acid) that resulted in FICI ≤ 0.5 , which is considered as synergistic interactions by the checkerboard test when using the pathogenic species *M. tuberculosis* (Tables 3 & 4). The combination of A23187 and tebipenem has the lowest FICI.

Table 3. MICs ($\mu\text{g/ml}$) of beta-lactams in *M. tuberculosis*

		Nigericin ^b	A23187 ^c
Amoxicillin	12.5	6.25	6.25
Amoxicillin+clavulanic acid ^a	1.25	0.625	0.625
Meropenem	5	2.5	5
Meropenem+clavulanic acid ^a	2.5	1.25	1.25
Faropenem	2.5	1.25	1.25
Faropenem+clavulanic acid ^a	2.5	1.25	1.25
Tebipenem	2.5	1.25	1.25
Tebipenem+clavulanic acid ^a	1.25	0.31	0.31

^aThe concentration of clavulanic acid was 2.5 µg/ml. ^bMICs of beta-lactams in chequerboard assay with nigericin. ^cMICs of beta-lactams in chequerboard assay with A23187.

Table 4. FICI index of combinations between ionophores and beta-lactams by chequerboard assay in *M. tuberculosis*

	Nigericin	A23187
Amoxicillin	1	1
Amoxicillin+clavulanic acid ^a	0.75	0.75
Meropenem	1	1.5
Meropenem+clavulanic acid ^a	0.75	1
Faropenem	1	1
Faropenem+clavulanic acid ^a	0.75	0.75
Tebipenem	1	0.75
Tebipenem+clavulanic acid ^a	0.5	0.375

^aThe concentration of clavulanic acid was 2.5 mg/L.

A23187 enhance the activity of beta-lactam against intracellular *M. tuberculosis* at nontoxic concentrations

We used 0.08 (one-quarter the IC₅₀) and 0.16 mg/L A23187 (half the IC₅₀) to identify the synergistic effect. As shown in Figure 2a and 2c, the log₁₀ CFU/ml of A23187 (0.08 mg/L) and tebipenem/clavulanate (0.625 or 1.25 mg/L) combination was lower than used alone. Figure 2b and 2d showed that the log₁₀ CFU/ml of A23187 (0.16 mg/L) and tebipenem/clavulanate (0.625 or 1.25 mg/L) combination was moderately lower than used alone. We can conclude that the combination of A23187 with tebipenem/clavulanate showed increased inhibition of bacterial viability than in absence of A23187.

Discussion

Nigericin and salinomycin are lipophilic compounds that can form complexes with the metal cations and, to a lesser extent, K⁺ and Na⁺ occurring with H⁺ exchange due to the proton motive force, modifying ion homeostasis. A23187 (calcimycin) is specific for divalent cations, such as Mn²⁺ and Ca²⁺. Nigericin and A23187 showed significant antimicrobial activity against *M. aurum*, *M. smegmatis*, *M. marinum* and the pathogenic slow-growing *M. tuberculosis* complex strains.

Investigation of the interaction of ionophores nigericin and A23187 with other antibiotics has led to the discovery of a synergy between these molecules and the

antibiotics of the family of beta-lactams. Synergism was observed with the combination of nigericin and meropenem by time-kill assays, A23187 and tebipenem by chequerboard assays. Meropenem was chosen because clinical trials showed its efficacy in the treatment of tuberculosis in contrast to other beta-lactams.

5 Our result showed that A23187 enhance the activity of beta-lactam against intracellular bacteria at concentrations that are not toxic for the macrophages. Another investigation showed that adding calcium ionophore A23187 to *M. tuberculosis* infected macrophage could increase calcium flux into the cells which is thought to block necrosis, stabilize mitochondrial permeability transition (MPT), decrease mitochondrial
10 cytochrome release, lower caspase activation, and accompany effective anti-mycobacterial activity. Therefore, A23187 could be an adjunct for beta-lactam against *M. tuberculosis*.

 In conclusion, we identified that Nigericin and A23187 exhibited synergistic interactions with some beta-lactams. This opens the way to use these antibiotics
15 combined with clavulanic acid or other inhibitors of beta-lactamase, cephalosporinase, or carbapenemase activity, together with ionophores, such as A23187, for the treatment of MDR-TB and other infectious bacterial diseases by oral administration.

CLAIMS

1. A combination for the inhibition of *Mycobacterium tuberculosis* complex (MTBC) strains multiplication and the treatment of tuberculosis, comprising:
 - 5 (a) an ionophore; and
 - (b) a β -lactam antibiotic.
2. The combination according to claim 1, wherein the ionophore is selected from the group consisting of monensin (also known as A-3823A), narasin A (also known as A-28086A), narasin B(also known as A-28086B), narasin D (also known as A-28086D), lasalocid, salinomycin, and maduramicin, alborixin (also known as S-14750A, CP-38,986), laidlomycin (also known as AB-78), lenoremycin (also known as A-130A, Ro21-6150), A-130B, A-130C, dianemycin (also known as A-150, M5-16183), A-204A, A-204B, Ionomycin (also known as A-218), deoxylaidlomycin (also known as A-712), calcimycin (also known as A-23187), septamycin (also known as BL-580 α and A-28695A), A-28695B, K-41A (also known as A-32887), septamycin (also known as BL-580ab), BL-580 β , BL-580 δ , BL-580Z, carriomycin, cationomycin, chloronoboritomycin A (also known as X-14766A), etheromycin (also known as CP-38,295, C 20-12, T-40517), deoxy-salinomycin (also known as SY-1), deoxy-epi-salinomycin(SY-2), deoxy-narasin, deoxy-epi-narasin, dianemycon (also known as M5-16183, A-150), emericid (also known as Ionomycin A and DE 3938), duamycin (also known as nigericin, helixin C, and azalomycin M), gridorixin, ionomycin, K-41B, lasalocid A (X-537A), lasalocid B, lasalocid C, lasalocid D, lasalocid E, iso-lasalocid A, leuseramycin, lomomycin B, lomomycin C, lysocellin, M-139603, monensin B, monensin C, monensin D, mutalomycin, noboritomycin A, noboritomycin B, RP 30504, RP 37454, salinomycin, salinomycin All, SY-4, SY-5, SY-8, tetronomycin, TM-531B, TM-531C, X-206, X-14547A, X-14667A, X-14667B, X-14868A, X-14868B, X-14868C, X-14868D, 5057, 6016, preferably selected from the group consisting of nigericin, A23187, salinomycin, and the combination thereof.
- 30 3. The combination according to claim 1 or 2, wherein the β -lactam antibiotic is selected from the group consisting of penicillins (e.g. temocillin, piperacillin), penems, and monobactams, carbapenems (e.g. meropenem, faropenem, imipenem,

- ertapenem, doripenem, panipenem/betamipron and biapenem as well as razupenem, tebipenem, lenapenem and tomopenem), ureidopenicillins (e.g. piperacillin), carbacephems (e.g. loracarbef) and cephalosporins (e.g. cefpodoxime, ceftazidime, cefotaxime, ceftriaxone, ceftobiprole, and ceftaroline), preferably is selected from the group consisting of carbapenem or penicillins antibiotic, more preferably the β -lactam antibiotic is selected from the group consisting of meropenem, amoxicillin and tebipenem.
- 5 the group consisting of carbapenem or penicillins antibiotic, more preferably the β -lactam antibiotic is selected from the group consisting of meropenem, amoxicillin and tebipenem.
4. The combination according to any one of claims 1-3, wherein the weight ratio of the ionophore and the β -lactam antibiotic is from 1:100 to 100:1, preferably 1:20 to 20:1, more preferably 1:5 to 5:1, more preferably 1:1.
- 10 20:1, more preferably 1:5 to 5:1, more preferably 1:1.
5. The combination according to any one of claims 1-3 further comprising (c) β -lactamase inhibitor, preferably the β -lactamase inhibitor is selected from the group consisting of Clavulanic Acid, Sulbactam, Sultamicillin, Tazobactam, Cilastatin, and Betamipron, preferably potassium clavulanate.
- 15 6. The combination according to claim 5, wherein the weight ratio of the ionophore, the β -lactam antibiotic and the β -lactamase inhibitor is from 1:100:1000 to 100:1:10, more preferably 1:20:500 to 20:1:25, most preferably 4:1:250.
7. A pharmaceutical composition comprising:
- 20 (a) an ionophore;
- (b) a β -lactam antibiotic; and optionally
- (c) a pharmaceutically acceptable carrier.
8. The composition according to claim 7, wherein the ionophore is selected from the group consisting of monensin (also known as A-3823A), narasin A (also known as A-28086A), narasin B (also known as A-28086B), narasin D (also known as A-28086D), lasalocid, salinomycin, and maduramicin, alborixin (also known as S-14750A, CP-38,986), laidlomycin (also known as AB-78), lenoremycin (also known as A-130A, Ro21-6150), A-130B, A-130C, dianemycin (also known as A-150, M5-16183), A-204A, A-204B, Ionomycin (also known as A-218), deoxylaidlomycin (also known as A-712), calcimycin (also known as A-23187), septamycin (also known as BL-580 α and A-28695A), A-28695B, K-41A (also known as A-32887), septamycin (also known as BL-580ab), BL-580 β , BL-580 δ , BL-580Z, carriomycin, cationomycin, chloronoboritomycin A (also known as
- 25 A-28086D), lasalocid, salinomycin, and maduramicin, alborixin (also known as S-14750A, CP-38,986), laidlomycin (also known as AB-78), lenoremycin (also known as A-130A, Ro21-6150), A-130B, A-130C, dianemycin (also known as A-150, M5-16183), A-204A, A-204B, Ionomycin (also known as A-218), deoxylaidlomycin (also known as A-712), calcimycin (also known as A-23187), septamycin (also known as BL-580 α and A-28695A), A-28695B, K-41A (also known as A-32887), septamycin (also known as BL-580ab), BL-580 β , BL-580 δ , BL-580Z, carriomycin, cationomycin, chloronoboritomycin A (also known as
- 30 BL-580Z, carriomycin, cationomycin, chloronoboritomycin A (also known as

- X-14766A), etheromycin (also known as CP-38,295, C 20-12, T-40517), deoxy-salinomycin (also known as SY-1), deoxy-epi-salinomycin (SY-2), deoxy-narasin, deoxy-epi-narasin, dianemycon (also known as M5-16183, A-150), emericid (also known as lonomycin A and DE 3938), duamycin (also known as nigericin, helixin C, and azalomycin M), gridorixin, ionomycin, K-41B, lasalocid A (X-537A), lasalocid B, lasalocid C, lasalocid D, lasalocid E, iso-lasalocid A, leuseramycin, lomomycin B, lomomycin C, lysocellin, M-139603, monensin B, monensin C, monensin D, mutalomycin, noboritomycin A, noboritomycin B, RP 30504, RP 37454, salinomycin, salinomycin All, SY-4, SY-5, SY-8, tetronomycin, TM-531B, TM-531C, X-206, X-14547A, X-14667A, X-14667B, X-14868A, X-14868B, X-14868C, X-14868D, 5057, 6016, preferably selected from the group consisting of nigericin, A23187, salinomycin, and the combination thereof.
9. The composition according to claim 7 or 8, wherein the β -lactam antibiotic is selected from the group consisting of penicillins (e.g. temocillin, piperacillin), penems, and monobactams, carbapenems (e.g. meropenem, faropenem, imipenem, ertapenem, doripenem, panipenem/betamipron and biapenem as well as razupenem, tebipenem, lenapenem and tomopenem), ureidopenicillins (e.g. piperacillin), carbacephems (e.g. loracarbef) and cephalosporins (e.g. cefpodoxime, ceftazidime, cefotaxime, ceftriaxone, ceftobiprole, and ceftaroline), preferably is selected the group consisting of carbapenem or penicillins antibiotic, more preferably the β -lactam antibiotic is selected from the group consisting of meropenem, amoxicillin and tebipenem.
10. The composition according to any one of claims 7-9, wherein the weight ratio of the ionophore and the β -lactam antibiotic is from 1:100 to 100:1, preferably 1:20 to 20:1, more preferably 1:5 to 5:1, most preferably 1:1.
11. The composition according to any of claims 7-9, further comprising β -lactamase inhibitor, preferably, the β -lactamase inhibitor is selected from the group consisting of Clavulanic Acid, Sulbactam, Sultamicillin, Tazobactam, Cilastatin, and Betamipron, preferably potassium clavulanate.
12. The composition according to claim 11, wherein the weight ratio of the ionophore, the β -lactam antibiotic and the β -lactamase inhibitor is from 1:100:1000 to 100:1:10, preferably 1:20:500 to 20:1:25, more preferably 4:1:250.

13. Use of the combination according to any one of claims 1-6 or the composition according to any of claims 7-12 in the manufacture of medicament for treatment of tuberculosis or for inhibiting MTBC multiplication.
14. A method for treating tuberculosis or for inhibiting MTBC strains multiplication,
5 comprising administering the combination according to any one of claims 1-6 or the composition according to any one of claims 7-12 to a subject in need thereof.
15. The use according to claim 13 or The method according to claim 14, wherein the MTBC strains are selected from the group consisting of drug-resistant MTBC strains, multidrug-resistant MTBC strains, extensively drug-resistant MTBC strains
10 and the combination thereof.
16. A kit, comprising:
- i) a first compartment containing an ionophore or a medicament comprising an ionophore;
 - ii) a second compartment containing a β -lactam antibiotic or a medicament
15 comprising a β -lactam antibiotic; and
 - iii) instructions for use in treating tuberculosis and/or for inhibiting *Mycobacterium tuberculosis* complex strains multiplication.
17. The kit according to claim 16, further comprising:
- iv) a third compartment containing a β -lactamase inhibitor or a medicament
20 comprising a β -lactamase inhibitor.

Figure 1

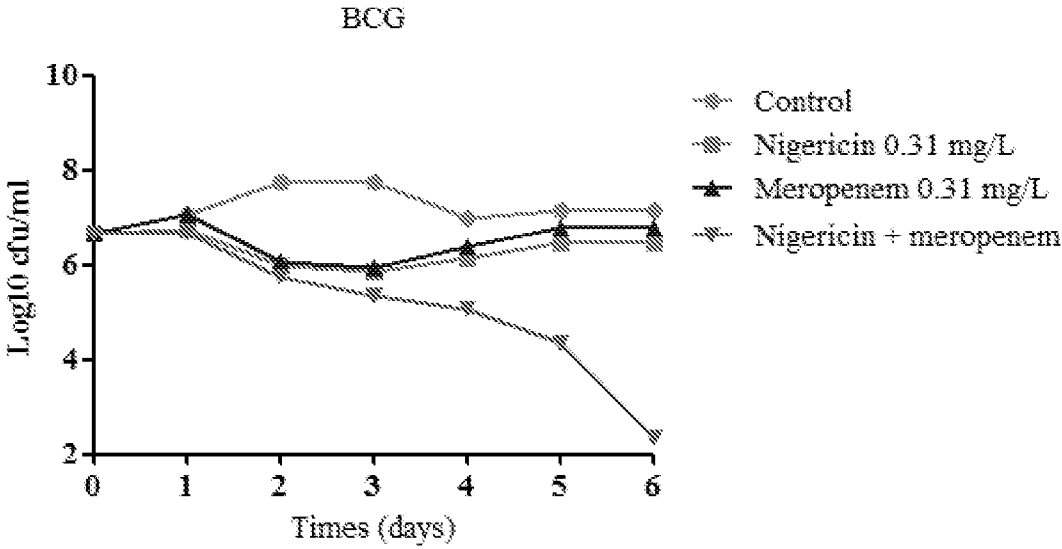
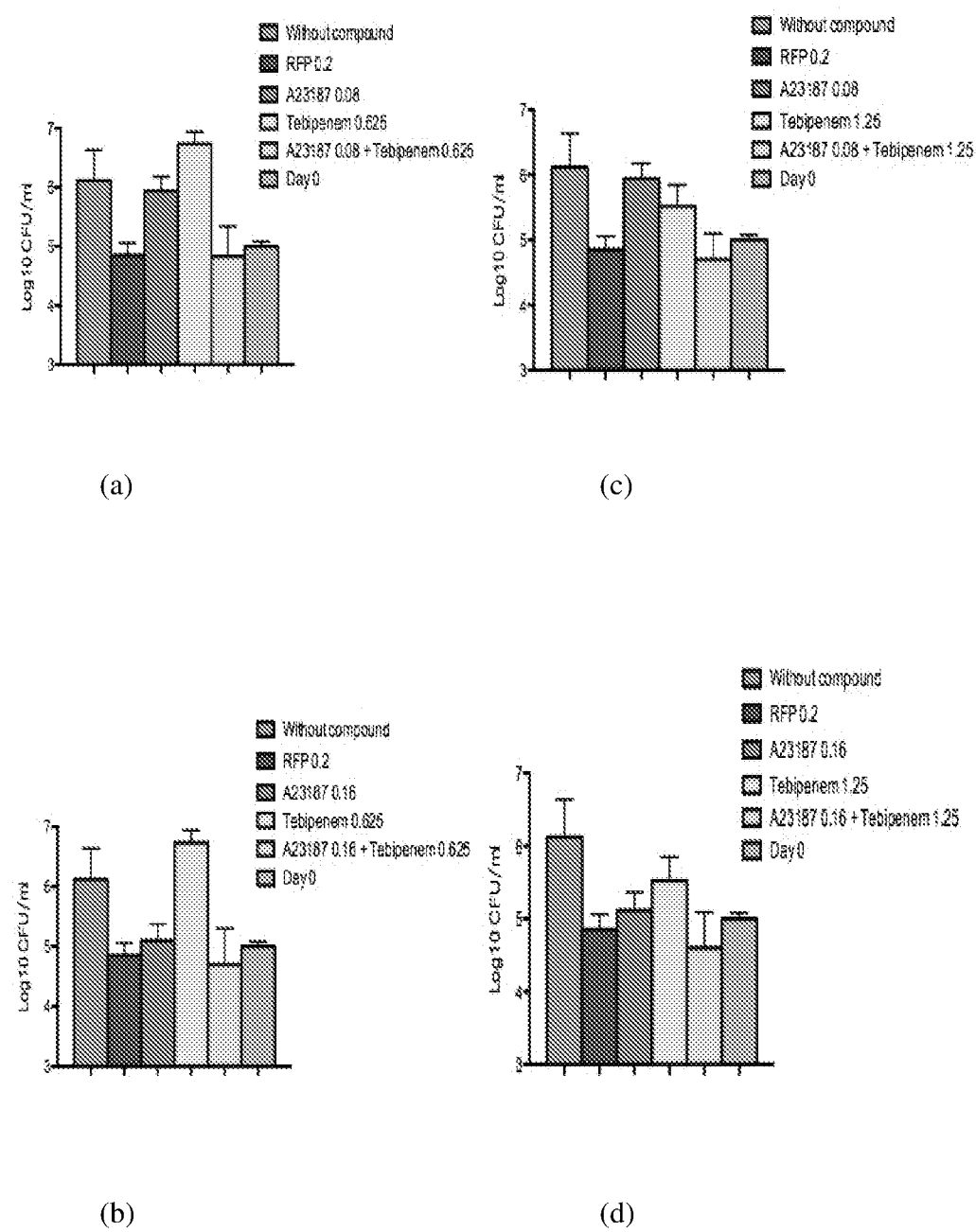


Figure 2



INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2017/087583

A. CLASSIFICATION OF SUBJECT MATTER

A61K 45/06(2006.01)i; A61K 31/351(2006.01)i; A61K 31/423(2006.01)i; A61K 31/43(2006.01)i; A61K 31/424(2006.01)i; A61P 31/06(2006.01)i

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; A61P

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)

CNXTX, CNABS, CPRSABS, CNMED, CNKI, DWPI, SIPOABS, EPODOC, JPABS, KRABS, CJFD, MEDNPL, SIPONPL, CSCD, CAPLUS, Pubmed, Embase: ionophore, polyether antibiotics, A-23187, nigericin, salinomycin, monensin, A-3823A, narasin, A-28086A, A-28086B, A-28086D, lasalocid, maduramicin, alborixin, S-14750A, laidlomycin, lenoremeycin, dianemeycin, ionomycin, deoxylaidlomycin, calcimycin, A-28695A, A-28695B, K-41A, A-32887, septamycin, carriomycin, cationomycin, chloronoboritomycin, X-14766A, etheromycin, T-40517, dianemycon, M5-16183, emericid, lonomycin, duamycin, helixin C, azalomycin, gridorixin, ionomycin, K-41B, lasalocid, leuseramycin, lomomycin, lysocellin, M-139603, mutalomycin, noboritomycin, RP 30504, RP 37454, tetronomycin, cillin, cefa, penem, cephamycin, cefto, carbopenem, monobactam, carbacephem, cephalosporin, lactam, tuberculosis, MTBC, tubercle bacillus

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2013103780 A1 (TRUSTEES OF BOSTON UNIVERSITY) 11 July 2013 (2013-07-11) see claims 1-5, 11, 22-34, 43-51, description, paragraphs [00196], [00256], [00297-00299]	1-17
Y	WO 2013103780 A1 (TRUSTEES OF BOSTON UNIVERSITY) 11 July 2013 (2013-07-11) see claims 1-5, 11, 22-34, 43-51, description, paragraphs [00196], [00256], [00297-00299]	2-6, 8-15
X	WO 2014121343 A1 (LUODA PHARMA PTY LTD., ET AL.) 14 August 2014 (2014-08-14) see claims 14, 18, 21, 30, description, paragraphs [0050], [0055], [0060]	1-3, 5, 7-9, 11



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

“L” document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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

“P” document published prior to the international filing date but later than the priority date claimed

“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

28 August 2017

Date of mailing of the international search report

14 September 2017

Name and mailing address of the ISA/CN

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2017/087583

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SALA, Claudia, et al. "Simple Model for Testing Drugs against Nonreplicating Mycobacterium tuberculosis" <i>ANTIMICROBIAL AGENTS AND CHEMOTHERAPY</i> , Vol. 54, No. 10, 02 August 2010 (2010-08-02), ISSN: 1098-6596, see abstract, page 4156 paragraph 1, fig. 6	2-6, 8-15

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2017/087583

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: **14-15**
because they relate to subject matter not required to be searched by this Authority, namely:

[1] The subject matter of claims 14-15 relates to a method of treatment of the human or animal body by therapy and therefore does not warrant an international search according to the criteria set out in Rule 39.1(iv). Thus, the search is based on the subject matter that could reasonably be expected to be claimed later in the procedure, and the corresponding claims, namely use of the combination according to any one of claims 1-6 or the composition according to any of claims 7-12 in the manufacture of medicament for treatment of tuberculosis or for inhibiting MTBC multiplication.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.

PCT/CN2017/087583

Patent document cited in search report			Publication date (day/month/year)	Patent family member(s)			Publication date (day/month/year)
WO	2013103780	A1	11 July 2013	US	2015071904	A1	12 March 2015
WO	2014121343	A1	14 August 2014	US	2015366797	A1	24 December 2015
				RU	2015132211	A	16 March 2017
				AU	2014214548	A1	20 August 2015
				EP	2953624	A1	16 December 2015
				JP	2016507533	A	10 March 2016
				CN	105492005	A	13 April 2016
				MX	2015010191	A	03 August 2016
				CA	2900373	A1	14 August 2014
				KR	20150115837	A	14 October 2015
				ZA	201506652	A	22 February 2017
				WO	2014121343	A9	14 August 2014
				IN	201502262	P3	27 May 2016