Title: COMBINATION COMPRISING PIPERINE AND POLYMXYINS FOR TREATING MICROBIAL INFECTIONS

Abstract: The invention provides a combination comprising piperine or a pharmaceutically acceptable derivative or stereoisomer thereof, and a polymyxin selected from polymyxin B and colistin, or a pharmaceutically acceptable derivative thereof. This combination is particularly useful for the treatment of microbial infections.
COMBINATION COMPRISING PIPERINE AND POLYMYXINS FOR TREATING MICROBIAL INFECTIONS

Field of the Invention

The present invention relates to the combination of piperine or a pharmaceutically acceptable derivative or stereoisomer thereof with a polymyxin selected from colistin and polymyxin B or a pharmaceutically acceptable derivative thereof, and the use of this combination for the treatment of microbial infections. In particular, it relates to the use of such combinations to kill multiplying microorganisms associated with microbial infections.

Background

Before the introduction of antibiotics, patients suffering from acute infectious diseases (e.g. tuberculosis or pneumonia) had a low chance of survival. For example, mortality from tuberculosis was around 50%. Although the introduction of antimicrobial agents in the 1940s and 1950s rapidly changed this picture, bacteria have responded by progressively gaining resistance to commonly used antibiotics. Now, every country in the world has antibiotic-resistant bacteria. Indeed, more than 70% of bacteria that give rise to hospital acquired infections in the USA resist at least one of the main antimicrobial agents that are typically used to fight infection (Nature Reviews, Drug Discovery, 1, 895-910 (2002)).

One way of tackling the growing problem of resistant bacteria is the development of new classes of antimicrobial agents. However, until the introduction of linezolid in 2000, there had been no new class of antibiotic marketed for over 37 years. Moreover, even the development of new classes of antibiotic provides only a temporary solution, and indeed there are already reports of resistance of certain bacteria to linezolid (Lancet, 357, 1179 (2001) and Lancet, 358, 207-208 (2001)).

In order to develop more long-term solutions to the problem of bacterial resistance, it is clear that alternative approaches are required. One such alternative approach is to minimise, as much as is possible, the opportunities that bacteria are given for developing resistance to important antibiotics. Thus, strategies that can be adopted include limiting the use of antibiotics for the treatment of non-acute infections, as well as controlling which antibiotics are fed to animals in order to promote growth. However, in order to tackle the problem more effectively, it is necessary to gain an understanding of the actual mechanisms by which bacteria generate resistance to antibiotic agents. To do this requires first a consideration of how current antibiotic agents work to kill bacteria.
Antimicrobial agents target essential components of bacterial metabolism. For example, the β-lactams (e.g. penicillins and cephalosporins) inhibit cell wall synthesis, whereas other agents inhibit a diverse range of targets, such as DNA gyrase (quinolones) and protein synthesis (e.g. macrolides, aminoglycosides, tetracyclines and oxazolidinones). The range of organisms against which the antimicrobial agents are effective varies, depending upon which organisms are heavily reliant upon the metabolic step(s) that is/are inhibited. Further, the effect upon bacteria can vary from a mere inhibition of growth (i.e. a bacteriostatic effect, as seen with agents such as the tetracyclines) to full killing (i.e. a bactericidal effect, as seen, e.g. with penicillin).

Bacteria have been growing on Earth for more than 3 billion years and, in that time, have needed to respond to vast numbers of environmental stresses. It is therefore perhaps not surprising that bacteria have developed a seemingly inexhaustible variety of mechanisms by which they can respond to the metabolic stresses imposed upon them by antibiotic agents. Indeed, mechanisms by which the bacteria can generate resistance include strategies as diverse as inactivation of the drug, modification of the site of action, modification of the permeability of the cell wall, overproduction of the target enzyme and bypass of the inhibited steps. Nevertheless, the rate of resistance emerges to a particular agent has been observed to vary widely, depending upon factors such as the agent's mechanism of action, whether the agent's mode of killing is time- or concentration-dependent, the potency against the population of bacteria and the magnitude and duration of the available serum concentration.

It has been proposed (Science, 264, 388-393 (1994)) that agents that target single enzymes (e.g. rifampicin) are the most prone to the development of resistance. Further, the longer those suboptimal levels of antimicrobial agent are in contact with the bacteria, the more likely the emergence of resistance.

Moreover, it is now known that many microbial infections include sub-populations of bacteria that are phenotypically resistant to antimicrobials (J. Antimicrob. Chemother., 4, 395-404 (1988); J. Med. Microbiol., 38, 197-202 (1993); J. Bacteriol., 182, 1794-1801 (2000); ibid. 182, 6358-6365 (2000); ibid. 183, 6746-6751 (2001); FEMS Microbiol. Lett., 202, 59-65 (2001); and Trends in Microbiology, 13, 34-40 (2005)). There appear to be several types of such phenotypically resistant bacteria, including persistent, stationary-phase bacteria, as well as those in the depths of biofilms. However, each of these types is characterised by its low
rate of growth compared to log-phase bacteria under the same conditions. Nutritional starvation and high cell densities are also common characteristics of such bacteria.

Although resistant to antimicrobial agents in their slow-growing state, phenotypically resistant bacteria differ from those that are genotypically resistant in that they regain their susceptibility to antimicrobials when they return to a fast-growing state (e.g. when nutrients become more readily available to them).

The presence of phenotypically resistant bacteria in an infection leads to the need for prolonged courses of antimicrobial agents, comprising multiple doses. This is because the resistant, slowly multiplying bacteria provide a pool of "latent" organisms that can convert to a fast-growing state when the conditions allow (thereby effectively re-initiating the infection). Multiple doses over time deal with this issue by gradually killing off the "latent" bacteria that convert to "active" form.

However, dealing with "latent" bacteria by administering prolonged courses of antimicrobials poses its own problems. That is, prolonged exposure of bacteria to suboptimal concentrations of antimicrobial agent can lead to the emergence of genotypically resistant bacteria, which can then multiply rapidly in the presence of even high concentrations of the antimicrobial.

Long courses of antimicrobials are more likely to encourage the emergence of genotypic resistance than shorter courses on the grounds that non-multiplying bacterial will tend to survive and, interestingly, probably have an enhanced ability to mutate to resistance (Proc. Natl. Acad. Sci. USA, 92, 11736-11740 (1995); J. Bacteriol., 179, 6688-6691 (1997); and Antimicrob. Agents Chemother., 44, 1771-1777 (2000)).

In the light of the above, a new approach to combating the problem of bacterial resistance might be to select and develop antimicrobial agents on the basis of their ability to kill "latent" microorganisms. The production of such agents would allow, amongst other things, for the shortening of chemotherapy regimes in the treatment of microbial infections, thus reducing the frequency with which genotypical resistance arises in microorganisms.

There have been reports of an anti-retroviral drug, zidovudine being active as an antimicrobial when combined with gentamicin. International Patent Application published as WO2014/147405 describes the use of zidovudine in combination with a polymyxin selected
from colistin and polymyxin B for treating a microbial infection. Given the importance of antimicrobial agents such as polymyxins in the fight against bacterial infection, the identification of further agents capable of enhancing their anti-bacterial activity addresses an important need.

International Patent Application, Publication Number WO2000028074 describes a method of screening compounds to determine their ability to kill log phase (i.e. multiplying) and/or clinically latent microorganisms. Using this method, the Applicant has observed that many plant extracts, such as piperine, have a synergistic effect with certain antimicrobial agents such as polymyxin, against multiplying and/or clinically latent microorganisms.

The present invention is based on the unexpected finding that the combination of piperine or a pharmaceutically acceptable derivative or stereoisomer thereof, and a polymyxin selected from polymyxin B and colistin or a pharmaceutically acceptable derivative thereof exhibit synergistic antimicrobial activity against log phase (i.e. multiplying) and/or clinically latent microorganisms. Particularly against log phase bacteria. In other words, the combination has a greater biological activity than the expected additive effect of each agent at the stated dosage level. The surprising biological activity of the combinations of the present invention offers the opportunity to shorten chemotherapy regimens and may result in a reduction in the emergence of microbial resistance associated with the use of such combinations.

Synergy in the context of antimicrobial drugs is measured in a number of ways that conform to the generally accepted opinion that “synergy is an effect greater than additive”. One of the ways to assess whether synergy has been observed is to use the “chequerboard” technique. This is a well-accepted method that leads to the generation of a value called the fractional inhibitory concentration index (FICI). Orhan et al J. Clin. Microbiol. 2005, 43(1):140 describes the chequerboard method and analysis in the paragraph bridging pages 140-141, and explains that the FICI value is a ratio of the sum of the MIC (Minimum Inhibitory Concentration) level of each individual component alone and in the mixture. The combination is considered synergistic when the ΣFIC is <0.5, indifferent when the ΣFIC is >0.5 to <2, and antagonistic when the ΣFIC is >2.

Another accepted test for ascertaining the presence or absence of synergy is to use time-kill methods where the dynamic effect of a drug combination is compared to each drug alone when assessing the effect on bacterial log or stationary-growth over time. Again, the possible results are for synergistic, additive or antagonistic effects.
Summary of the Invention

Thus, in one embodiment the present invention provides a combination of piperine or a pharmaceutically acceptable derivative or stereoisomer thereof and a polymyxin selected from polymyxin B and colistin or a pharmaceutically acceptable derivative thereof.

In another embodiment the present invention provides the use of piperine or a pharmaceutically acceptable derivative or stereoisomer thereof in combination with a polymyxin selected from colistin or polymyxin B or a pharmaceutically acceptable derivative thereof, in the manufacture of a medicament for treating a microbial infection.

Additionally the present invention provides the combination of piperine or a pharmaceutically acceptable derivative or stereoisomer thereof and a polymyxin selected from polymyxin B and colistin or a pharmaceutically acceptable derivative thereof, for use in the treatment of a microbial infection, preferably for use in the treatment of a bacterial infection.

In a further embodiment, the invention provides a method of treating a microbial infection which comprises administering to a mammal, including man, piperine or a pharmaceutically acceptable derivative or stereoisomer thereof in combination with a polymyxin selected from colistin or polymyxin B or a pharmaceutically acceptable derivative thereof.

There is also provided a pharmaceutical composition comprising piperine or a pharmaceutically acceptable derivative or stereoisomer thereof in combination with a polymyxin selected from colistin or polymyxin B or a pharmaceutically acceptable derivative thereof, and a pharmaceutically acceptable adjuvant, diluent or carrier. In one embodiment the pharmaceutical composition is for use in the treatment of a microbial infection, preferably wherein the microbial infection is a bacterial infection.

In a further embodiment, the invention relates to a product comprising piperine or a pharmaceutically acceptable derivative or stereoisomer thereof in combination with a polymyxin selected from colistin or polymyxin B or a pharmaceutically acceptable derivative thereof, as a combined preparation for simultaneous, separate or sequential use in killing multiplying and/or clinically latent microorganisms associated with a microbial infection. Preferably for killing multiplying bacteria associated with a bacterial infection.
**Brief Description of the Drawings**

**Figure 1** is a time-kill curve (Log CFU/ml against time (hours)) for colistin in the form of colistimethate sodium at a concentration of 32 mg/ml, piperine at a concentration of 256 mg/ml and the combination of colistin (32 mg/ml) and piperine (256 mg/ml).

**Figure 2** is a time-kill curve (Log CFU/ml against time (hours)) for colistin in the form of colistimethate sodium at a concentration of 32 mg/ml, piperine at a concentration of 128 mg/ml and the combination of colistin (32 mg/ml) and piperine (128 mg/ml).

**Figure 3** is a time-kill curve (Log CFU/ml against time (hours)) for colistin in the form of colistimethate sodium at a concentration of 32 mg/ml, piperine at a concentration of 64 mg/ml and the combination of colistin (32 mg/ml) and piperine (64 mg/ml).

**Figure 4** is a time-kill curve (Log CFU/ml against time (hours)) for colistin in the form of colistimethate sodium at a concentration of 16 mg/ml, piperine at a concentration of 256 mg/ml and the combination of colistin (16 mg/ml) and piperine (256 mg/ml).

**Detailed Description of the Invention**

As described below, the combinations of the present invention have been demonstrated to be particularly effective against drug-resistant bacteria, particularly drug-resistant Gram-negative bacteria, opening the way for said combinations to be administered both to drug-resistant strains and in said strains before drug-resistance is built up, i.e. as a first line treatment.

As used herein, the term “in combination with” covers both separate and sequential administration of the piperine and the polymyxin. When the piperine and polymyxin are administered sequentially, either the piperine or the polymyxin may be administered first. When administration is simultaneous, the piperine and polymyxin may be administered either in the same or a different pharmaceutical composition. Adjunctive therapy, i.e. where one agent is used as a primary treatment and the other agent is used to assist that primary treatment, is also an embodiment of the present invention.

The combinations of the present invention may be used to treat microbial infections. In particular they may be used to kill multiplying and/or clinically latent microorganisms associated with microbial infections, preferably multiplying microorganisms associated with
microbial infections, e.g. multiplying bacteria associated with Gram-negative bacterial infections. References herein to the treatment of a microbial infection therefore include killing multiplying and/or clinically latent microorganisms associated with such infections.

As used herein, "kill" means a loss of viability as assessed by a lack of metabolic activity.

As used herein, "clinically latent microorganism" means a microorganism that is metabolically active but has a growth rate that is below the threshold of infectious disease expression. The threshold of infectious disease expression refers to the growth rate threshold below which symptoms of infectious disease in a host are absent.

The metabolic activity of clinically latent microorganisms can be determined by several methods known to those skilled in the art; for example, by measuring mRNA levels in the microorganisms or by determining their rate of uridine uptake. In this respect, clinically latent microorganisms, when compared to microorganisms under logarithmic growth conditions (in vitro or in vivo), possess reduced but still significant levels of:

(I) mRNA (e.g. from 0.0001 to 50%, such as from 1 to 30, 5 to 25 or 10 to 20%, of the level of mRNA); and/or

(II) uridine (e.g. [³H]uridine) uptake (e.g. from 0.0005 to 50%, such as from 1 to 40, 15 to 35 or 20 to 30% of the level of [³H]uridine uptake).

Clinically latent microorganisms typically possess a number of identifiable characteristics. For example, they may be viable but non-culturable; i.e. they cannot typically be detected by standard culture techniques, but are detectable and quantifiable by techniques such as broth dilution counting, microscopy, or molecular techniques such as polymerase chain reaction. In addition, clinically latent microorganisms are phenotypically tolerant, and as such are sensitive (in log phase) to the biostatic effects of conventional antimicrobial agents (i.e. microorganisms for which the minimum inhibitory concentration (MIC) of a conventional antimicrobial is substantially unchanged); but possess drastically decreased susceptibility to drug-induced killing (e.g. microorganisms for which, with any given conventional antimicrobial agent, the ratio of minimum microbiocidal concentration (e.g. minimum bactericidal concentration, MBC) to MIC is 10 or more).

As used herein, the term "microorganisms" means fungi and bacteria. References herein to "microbial", "antimicrobial" and "antimicrobially" shall be interpreted accordingly. For
example, the term "microbial" means fungal or bacterial, and "microbial infection" means any fungal or bacterial infection.

In one embodiment of the invention, one or more of the aforementioned combinations is used to treat a bacterial infection, in particular the combinations may be used to kill clinically latent microorganisms associated with a bacterial infection. As used herein, the term "bacteria" (and derivatives thereof, such as "microbial infection") includes, but is not limited to, references to organisms (or infections due to organisms) of the following classes and specific types:

Gram-positive cocci, such as Staphylococci (e.g. Staph. aureus, Staph. epidermidis, Staph. saprophyticus, Staph. auricularis, Staph. capitis capitis, Staph. c. ureolyticus, Staph. caprae, Staph. cohnii cohnii, Staph. c. urealyticus, Staph. equorum, Staph. gallinarum, Staph. haemolyticus, Staph. hominis hominis, Staph. h. novobiosepticus, Staph. hyicus, Staph. intermedius, Staph. lugdunensis, Staph. pasteuri, Staph. saccharolyticus, Staph. schleiferi schleiferi, Staph. s. coagulans, Staph. scuri, Staph. simulans, Staph. warneri and Staph. xylosus);

Streptococci (e.g.beta-haemolytic, pyogenic streptococci (such as Strept. agalactiae, Strept. canis, Strept. dysgalactiae dysgalactiae, Strept. dysgalactiae equisimilis, Strept. equi equi, Strept. equi zooepidemicus, Strept. iniae, Strept. porcinus and Strept. pyogenes), microaerophilic, pyogenic streptococci (Streptococcus "milleri", such as Strept. anginosus, Strept. constellatus constellation, Strept. constellatus pharyngidis and Strept. intermedius), oral streptococci of the "mitis" (alpha-haemolytic - Streptococcus "viridans", such as Strept. mitis, Strept. oralis, Strept. sanguinis, Strept. cristatus, Strept. gordonii and Strept. parasanguinis), "salivarius" (non-haemolytic, such as Strept. salivarius and Strept. vestibularis) and "mutans" (tooth-surface streptococci, such as Strept. criceti, Strept. mutans, Strept. ratti and Strept. sobrinus) groups, Strept. acediminus, Strept. bovis, Strept. faecalis, Strept. equinus, Strept. pneumoniae and Strept. suis, or Streptococci alternatively classified as Group A, B, C, D, E, G, L, P, U or V Streptococcus);

Gram-negative cocci, such as Neisseria gonorrhoeae, Neisseria meningitidis, Neisseria cinerea, Neisseria elongata, Neisseria flavescens, Neisseria lactamica, Neisseria mucosa, Neisseria sicca, Neisseria subflava and Neisseria weaveri;

Bacillaceae, such as Bacillus anthracis, Bacillus subtilis, Bacillus thuringiensis, Bacillus stearothermophilus and Bacillus cereus;

Enterobacteriaceae, such as Escherichia coli,
Enterobacter (e.g. Enterobacter aerogenes, Enterobacter agglomerans and Enterobacter cloacae), Citrobacter (such as Citrobacter freundii and Citrobacter diversus), Hafnia (e.g. Hafnia alvei), Erwinia (e.g. Erwinia persicinus), Morganella morganii, Salmonella (Salmonella enterica and Salmonella typhi), Shigella (e.g. Shigella dysenteriae, Shigella flexneri, Shigella boydii and Shigella sonnei), Klebsiella (e.g. Klebsiella pneumoniae, Klebsiella oxytoca, Klebsiella ornitholytica, Klebsiella planticola, Klebs. ozaenae, Klebs. terrigena, Klebs. granulomatis (Calymmatobacterium granulomatis) and Klebs. rhinoscleromatis), Proteus (e.g. Pr. mirabilis, Pr. rettgeri and Pr. vulgaris), Providencia (e.g. Providencia alcalifaciens, Providencia rettgeri and Providencia stuartii), Serratia (e.g. Serratia marcescens and Serratia liquefaciens), and Yersinia (e.g. Yersinia enterocolitica, Yersinia pestis and Yersinia pseudotuberculosis);

Enterococci (e.g. Enterococcus avium, Enterococcus casseliflavus, Enterococcus cecorum, Enterococcus dispar, Enterococcus durans, Enterococcus faecalis, Enterococcus faecium, Enterococcus flavescens, Enterococcus gillii, Enterococcus hirae, Enterococcus melodoratus, Enterococcus mundtii, Enterococcus pseudoavium, Enterococcus raffinosus and Enterococcus solitarius);

Helicobacter (e.g. Helicobacter pylori, Helicobacter cinaedi and Helicobacter fennelliae);

Acinetobacter (e.g. A. baumannii, A. calcoaceticus, A. haemolyticus, A. johnsonii, A. junii, A. lwoffii and A. radioresistens);

Pseudomonas (e.g. Ps. aeruginosa, Ps. maltophilia (Stenotrophomonas maltophilia), Ps. alcaligenes, Ps. chlororaphis, Ps. fluorescens, Ps. luteola, Ps. mendocina, Ps. monteilii, Ps. oryzihabitans, Ps. pertocinogena, Ps. pseucaaligenes, Ps. putida and Ps. stutzeri);

Bacteroides fragilis;

Peptococcus (e.g. Peptococcus niger); Peptostreptococcus;


Mycoplasma (e.g. M. pneumoniae, M. hominis, M. genitalium and M. urealyticum);

Mycobacteria (e.g. Mycobacterium tuberculosis, Mycobacterium avium, Mycobacterium fortuitum, Mycobacterium marinum, Mycobacterium kansasii, Mycobacterium chelonae, Mycobacterium abscessus, Mycobacterium leprae, Mycobacterium smegmatis, Mycobacterium africanum, Mycobacterium alvei, Mycobacterium asiaticum, Mycobacterium aurum, Mycobacterium bohemicum, Mycobacterium bovis, Mycobacterium branderi,
Mycobacterium brumae, Mycobacterium celatum, Mycobacterium chubense, Mycobacterium confluentis, Mycobacterium conspicuum, Mycobacterium cookii, Mycobacterium flavescentis, Mycobacterium gadi, Mycobacterium gastri, Mycobacterium genavense, Mycobacterium gordiae, Mycobacterium goodii, Mycobacterium haemophilum, Mycobacterium hessicum, Mycobacterium intracellulare, Mycobacterium interjectum, Mycobacterium heidelbergense, Mycobacterium lentiflavum, Mycobacterium malmoense, Mycobacterium microgenicum, Mycobacterium microti, Mycobacterium mucogenicum, Mycobacterium neoaurum, Mycobacterium nonchromogenicum, Mycobacterium peregrinum, Mycobacterium phlei, Mycobacterium scrofulaceum, Mycobacterium shimoidei, Mycobacterium simulii, Mycobacterium szulgai, Mycobacterium terrae, Mycobacterium thermoresistabile, Mycobacterium triplex, Mycobacterium triviale, Mycobacterium tusciae, Mycobacterium ulcerans, Mycobacterium vaccae, Mycobacterium wolinskyi and Mycobacterium xenopi; Haemophilus (e.g. Haemophilus influenzae, Haemophilus ducreyi, Haemophilus aegyptius, Haemophilus parainfluenzae, Haemophilus haemolyticus and Haemophilus parahaemolyticus); Actinobacillus (e.g. Actinobacillus actinomycetemcomitans, Actinobacillus equuli, Actinobacillus hominis, Actinobacillus lignieresii, Actinobacillus suis and Actinobacillus ureae); Actinomyces (e.g. Actinomyces israelii); Brucella (e.g. Brucella abortus, Brucella canis, Brucella melitensis and Brucella suis); Campylobacter (e.g. Campylobacter jejuni, Campylobacter coli, Campylobacter lari and Campylobacter fetus); Listeria monocytogenes; Vibrio (e.g. Vibrio cholerae and Vibrio parahaemolyticus, Vibrio alginolyticus, Vibrio carchariae, Vibrio fluvialis, Vibrio furnissii, Vibrio hollisae, Vibrio metschnikovii, Vibrio mimicus and Vibrio vulnificus); Erysipelothrix rhusopathiae; Corynebacteriaceae (e.g. Corynebacterium diptheriae, Corynebacterium jeikeium and Corynebacterium urealyticum); Spirochaetaceae, such as Borrelia (e.g. Borrelia recurrentis, Borrelia burgdorferi, Borrelia afzelii, Borrelia andersonii, Borrelia bissettii, Borrelia garinii, Borrelia japonica, Borrelia lusitaniae, Borrelia turkii, Borrelia turdi, Borrelia valaisiana, Borrelia caucasia, Borrelia crocidurae, Borrelia duttonii, Borrelia graingeri, Borrelia hermsii, Borrelia hispanica, Borrelia latyschewii, Borrelia mazzottii, Borrelia parkeri, Borrelia persica, Borrelia liricatae and Borrelia venezuelensis) and Treponema (Treponema pallidum ssp. pallidum, Treponema pallidum ssp. endemicum, Treponema pallidum ssp. pertenue and Treponema carateum);
Pasteurella (e.g. Pasteurella aerogenes, Pasteurella bettyae, Pasteurella canis, Pasteurella dagmatis, Pasteurella gallinarum, Pasteurella haemolytica, Pasteurella multocida multocida, Pasteurella multocida gallicida, Pasteurella multocida septica, Pasteurella pneumotropica and Pasteurella stomatis);
Bordetella (e.g. Bordetella bronchiseptica, Bordetella hinzii, Bordetella holmseii, Bordetella parapertussis, Bordetella pertussis and Bordetella trematum);
Nocardiaeae, such as Nocardia (e.g. Nocardia asteroides and Nocardia brasiliensis);
Rickettsia (e.g. Rickettsia or Coxiella burnetii);
Legionella (e.g. Legionella anisa, Legionella birminghamensis, Legionella bozemani, Legionella cincinnatiensis, Legionella dumoffii, Legionella feeleei, Legionella gormanii, Legionella hackeliae, Legionella israelensis, Legionella jordanis, Legionella lansingensis, Legionella longbeachae, Legionella maceachernii, Legionella micdadei, Legionella oakridgensis, Legionella pneumophila, Legionella sainhelensi, Legionella tucsonensis and Legionella wadsworthii);
Moraxella catarrhalis;
Cyclospora cayetanensis; Entamoeba histolytica; Giardia lambia; Trichomonas vaginalis; Toxoplasma gondii; Stenotrophomonas maltophilia; Burkholderia cepacia; Burkholderia mallei and Burkholderia pseudomallei; Francisella tularensis; Gardnerella (e.g. Gardnerella vaginalis and Gardnerella mobiluncus); Streptobacillus moniliformis; Flavobacteriaceae, such as Capnocytophaga (e.g. Capnocytophaga canimorsus, Capnocytophaga cynodegmi, Capnocytophaga gingivalis, Capnocytophaga granulosa, Capnocytophaga haemolytica, Capnocytophaga ochracea and Capnocytophaga sputigena);
Bartonella (Bartonella bacilliformis, Bartonella clarridgeiae, Bartonella elizabethae, Bartonella henselae, Bartonella quintana and Bartonella vinsonii arupensis);
Leptospira (e.g. Leptospira biflexa, Leptospira borgpetersenii, Leptospira inadai, Leptospira interrogans, Leptospira kirschneri, Leptospira naguchii, Leptospira santarosai and Leptospira weilii);
Spirillum (e.g. Spirillum minus);
Baceteroides (e.g. Bacteroides cacaoe, Bacteroides capillosus, Bacteroides coagulans, Bacteroides distasonis, Bacteroides eggerthii, Bacteroides forsythus, Bacteroides fragilis, Bacteroides merdae, Bacteroides ovatus, Bacteroides putredinis, Bacteroides pyogenes, Bacteroides splanchnicus, Bacteroides stercoris, Bacteroides tectus, Bacteroides thetaiotaomicron, Bacteroides uniformis, Bacteroides ureolyticus and Bacteroides vulgatus);
Prevotella (e.g. Prevotella bivia, Prevotella buccae, Prevotella corporis, Prevotella dentalis (Mitsuokella dentalis), Prevotella denticola, Prevotella disiens, Prevotella enoeca, Prevotella heparinolytica, Prevotella intermedia, Prevotella loeschii, Prevotella melaninogenica,
Prevotella nigrescens, Prevotella oralis, Prevotella oralis, Prevotella oulora, Prevotella tannerae, Prevotella venoralis and Prevotella zoogloeiformans);
Porphyromonas (e.g. Porphyromonas asaccharolytica, Porphyromonas cagingivalis, Porphyromonas canoris, Porphyromonas canalis, Porphyromonas catonae, Porphyromonas circumdentaria, Porphyromonas crevioricanis, Porphyromonas endodontalis, Porphyromonas gingivalis, Porphyromonas gingivicanus, Porphyromonas levii and Porphyromonas macacae);
Fusobacterium (e.g. F. gonadiformans, F. mortiferum, F. naviforme, F. necrogenes, F. necrophorum necrophorum, F. necrophorum fundiforme, F. nucleatum nucleatum, F. nucleatum fusiforme, F. nucleatum polymorphum, F. nucleatum vincentii, F. periodonticum, F. russi, F. ulcerans and F. varium);
Chlamydia (e.g. Chlamydia trachomatis); Cryptosporidium (e.g. C. parvum, C. hominis, C. canis, C. felis, C. meleagris and C. muris); Chlamydophila (e.g. Chlamydophila abortus (Chlamydia psittaci), Chlamydophila pneumoniae (Chlamydia pneumoniae) and Chlamydophila psittaci (Chlamydia psittaci));
Leuconostoc (e.g. Leuconostoc citreum, Leuconostoc cremoris, Leuconostoc dextranicum, Leuconostoc lactis, Leuconostoc mesenteroides and Leuconostoc pseudomesenteroides);
Gemella (e.g. Gemella bergeri, Gemella haemolytica, Gemella morbillorum and Gemella sanguinis); and Ureaplasma (e.g. Ureaplasma parvum and Ureaplasma urealyticum).

Preferably, the bacterial infections treated by the combinations described herein are gram-negative bacterial infections. Particular Gram-negative bacteria that may be treated using a combination of the invention include:
Entero-bacteriaceae, such as Escherichia coli, Klebsiella (e.g. Klebs. pneumoniae and Klebs. oxytoca) and Proteus (e.g. Pr. mirabilis, Pr. rettgeri and Pr. vulgaris);
Haemophilis influenzae;
Mycobacteria, such as Mycobacterium tuberculosis; and
Enterobacter (e.g. Enterobacter cloacae).

Preferably, the bacteria are Entero-bacteriaceae, such as Escherichia coli, Klebsiella (e.g. Klebs. pneumoniae and Klebs. oxytoca) and Enterobacter (e.g. Enterobacter cloacae). Particularly preferred are Escherichia coli, Klebsiella and Enterobacter, e.g. Escherichia coli, Klebs. pneumoniae and Enterobacter cloacae.

The combination of the present invention is particularly beneficial in treating (multi)-drug-resistant ((M)DR) bacteria. With respect to Entero-bacteriaceae, drug resistance most often
builds up to carbapenemase i.e. carbapenemase-resistant strains and “extended spectrum β-lactamase” (ESBL) strains for example New Delhi Metallo-beta-lactamase-1 (NDM-1) resistant Klebs. Pneumonia, and NDM-1 E.coli.

It should be kept in mind that although a combination such as that claimed may initially be demonstrated to be functional in treating (M)DR strains, they can then be used in treating non-resistant strains. This is especially valuable in the context of the presently claimed combination where the primary therapy for Enterobacteriaceae, such as Escherichia coli, Klebsiella (e.g. Klebs. pneumoniae and Klebs. oxytoca) and Enterobacter (e.g. Enterobacter cloacae) are anti-microbial drugs that are expensive due to prevailing patent protection. The replacement of such “ethical” drugs by a combination of “generic” antibiotics is thought to be beneficial from a therapeutic perspective as well as financial/economic perspective in times where governments are seeking to reduce the cost of healthcare.

The combinations of the present invention may be used to treat infections associated with any of the above-mentioned bacterial organisms, and in particular they may be used for killing multiplying and/or clinically latent microorganisms associated with such an infection, e.g. a Gram-negative bacterial infection.

Particular conditions which may be treated using the combination of the present invention include those which are caused by Gram-negative bacteria such as abscesses, asthma, bacillary dysentery, bacterial conjunctivitis, bacterial keratitis, bacterial vaginosis, bone and joint infections, bronchitis (acute or chronic), brucellosis, burn wounds, cat scratch fever, cellulitis, chancroid, cholangitis, cholecystitis, cystic fibrosis, cystitis, nephritis, diffuse panbronchiolitis, dental caries, diseases of the upper respiratory tract, empymea, endocarditis, endometritis, enteric fever, enteritis, epididymitis, epiglottitis, eye infections, furuncles, gardnerella vaginitis, gastrointestinal infections (gastroenteritis), genital infections, gingivitis, gonorrhoea, granuloma inguinale, Haverhill fever, infected burns, infections following dental operations, infections in the oral region, infections associated with prostheses, intraabdominal abscesses, Legionnaire’s disease, leptospirosis, listeriosis, liver abscesses, Lyme disease, lymphogranuloma venerium, mastitis, mastoiditis, meningitis and infections of the nervous system, non-specific urethritis, ophthalmia (e.g. ophthalmia neonatorum), osteomyelitis, otitis (e.g. otitis externa and otitis media), orchitis, pancreatitis, paronychia, pelveoperitonitis, peritonitis, peritonitis with appendicitis, pharyngitis, pleural effusion, pneumonia, postoperative wound infections, postoperative gas gangrene, prostatitis, pseudo-membranous colitis, psittacosis, pyelonephritis, Q fever, rat-bite fever,
Ritter's disease, salmonellosis, salpingitis, septic arthritis, septic infections, septicaemia, systemic infections, tonsillitis, trachoma, typhoid, urethritis, urinary tract infections, wound infections; or infections with, *Escherichia coli*, *Klebs. pneumoniae*, *Klebs. oxytoca*, *Pr. mirabilis*, *Pr. rettgeri*, *Pr. vulgaris*, *Haemophilis influenzae*, *Enterococcus faecalis*, *Enterococcus faecium*, and *Enterobacter cloacae*.

It will be appreciated that references herein to “treatment” extend to prophylaxis as well as the treatment of established diseases or symptoms.

As used herein the term “pharmaceutically acceptable derivative” means:

(a) pharmaceutically acceptable salts; and/or
(b) solvates (including hydrates).

Suitable acid addition salts include carboxylate salts (e.g. formate, acetate, trifluoroacetate, propionate, isobutyrate, heptanoate, decanoate, caprate, caprylate, stearate, acrylate, caproate, propiolate, ascorbate, citrate, glucuronate, glutamate, glycolate, α-hydroxybutyrate, lactate, tartrate, phenylacetate, mandelate, phenylpropionate, phenylbutyrate, benzoate, chlorobenzoate, methylbenzoate, hydroxybenzoate, methoxybenzoate, dinitrobenzoate, o-acetoxybenzoate, salicylate, nicotinate, isonicotinate, cinnamate, oxalate, malonate, succinate, suberate, sebacate, fumarate, malate, maleate, hydroxymaleate, hippurate, phthalate or terephthalate salts), halide salts (e.g. chloride, bromide or iodide salts), sulfonate salts (e.g. benzenesulfonate, methyl-, bromo- or chloro-benzenesulfonate, xylenesulfonate, methanesulfonate, ethanesulfonate, propanesulfonate, hydroxyethanesulfonate, 1- or 2-naphthalene-sulfonate or 1,5-naphthalenedisulfonate salts) or sulfate, pyrosulfate, bisulfate, sulfite, bisulfite, phosphate, monohydrogenphosphate, dihydrogenphosphate, metaphosphate, pyrophosphate or nitrate salts, and the like.

The polymyxin is colistin or polymyxin B or a pharmaceutically acceptable derivative thereof. For example, colistin sulfate, colistimethate sodium, or polymyxin B sulfate. Particularly preferred is colistin, colistin sulfate or colistimethate sodium, e.g. colistin sulfate or colistimethate sodium. Most preferred is colistimethate sodium. Colistimethate sodium is also known in the art as colistin methanesulfonate sodium or colistin sulfomethate sodium.

Colistin is also known as polymyxin E and is an antibiotic produced by certain strains of the bacteria *Paenibacillus polymyxa*. Colistin has the following chemical structure and the IUPAC
chemical name N-(4-amino-1-(1-(4-amino-1-oxo-1-(3,12,23-tris(2-aminoethyl)-20-(1-hydroxyethyl)-6,9-diisobutyl-2,5,8,11,14,19,22-heptaoxo-1,4,7,10,13,18-hexaazacyclotricosan-15-ylamino)butan-2-ylamino)-3-hydroxybutan-2-ylamino)-1-oxobutan-2-yl)-N,N-dimethylheptanamide.

Colistin is available commercially as colistin sulfate and colistimethate sodium. Colistin sulfate is cationic and colistimethate sodium is anionic. Colistimethate sodium is also readily hydrolysed to a variety of methanesulfonated derivatives.

Piperine is the alkaloid responsible for the pungency of black pepper (Piper nigrum) and long pepper (Piper longum). Piperine has the following chemical structure and has the IUPAC Chemical name of 1-[5-(1,3-Benzodioxol-5-yl)-1-oxo-2,4-pentadienyl]piperidine.

Piperine may exist as stereoisomers. In particular piperine may exist as geometric isomers due to the cis/trans configuration of the C=C double bonds in the alkyl chain. The present invention thus contemplates the use of all the stereoisomers, particularly all geometric isomers of piperine. In particular, the invention contemplates the use the isomer chavicine. Chavicine is one of four diastereomeric geometric isomers of piperine and has the following structure:

Like piperine, chavicine is the alkaloid responsible for the pungency of black pepper and long pepper.
Compounds for use according to the invention may be administered as the raw material but the active ingredients are preferably provided in the form of pharmaceutical compositions.

The active ingredients may be used either as separate formulations or as a single combined formulation. When combined in the same formulation it will be appreciated that the two compounds must be stable and compatible with each other and the other components of the formulation.

Formulations of the invention include those suitable for oral, parenteral (including subcutaneous e.g. by injection or by depot tablet, intradermal, intrathecal, intramuscular e.g. by depot and intravenous), rectal and topical (including dermal, buccal and sublingual) or in a form suitable for administration by inhalation or insufflation administration. The most suitable route of administration may depend upon the condition and disorder of the patient.

Preferably, the compositions of the invention are formulated for oral or topical administration. In a preferred embodiment, the composition is a cream or an ointment adapted for nasal administration, in particular for delivery to the anterior nares.

The formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy e.g. as described in “Remington: The Science and Practice of Pharmacy”, Lippincott Williams and Wilkins, 21st Edition, (2005). Suitable methods include the step of bringing into association to active ingredients with a carrier which constitutes one or more excipients. In general, formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both and then, if necessary, shaping the product into the desired formulation. It will be appreciated that when the two active ingredients are administered independently, each may be administered by a different means.

When formulated with excipients, the active ingredients may be present in a concentration from 0.1 to 99.5% (such as from 0.5 to 95%) by weight of the total mixture; conveniently from 30 to 95% for tablets and capsules and 0.01 to 50% (such as from 3 to 50%) for liquid preparations.

Formulations suitable for oral administration may be presented as discrete units such as capsules, cachets or tablets (e.g. chewable tablets in particular for paediatric administration), each containing a predetermined amount of active ingredient; as powder or granules; as a solution or suspension in an aqueous liquid or non-aqueous liquid; or as an oil-in-water liquid
emulsion or water-in-oil liquid emulsion. The active ingredients may also be presented as a bolus, electuary or paste.

A tablet may be made by compression or moulding, optionally with one or more excipients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredient in a free-flowing form such as a powder or granules, optionally mixed with other conventional excipients such as binding agents (e.g. syrup, acacia, gelatin, sorbitol, tragacanth, mucilage of starch, polyvinylpyrrolidone and/or hydroxymethyl cellulose), fillers (e.g. lactose, sugar, microcrystalline cellulose, maize-starch, calcium phosphate and/or sorbitol), lubricants (e.g. magnesium stearate, stearic acid, talc, polyethylene glycol and/or silica), disintegrants (e.g. potato starch, croscarmellose sodium and/or sodium starch glycolate) and wetting agents (e.g. sodium lauryl sulphate). Moulded tablets may be made by moulding in a suitable machine a mixture of the powdered active ingredient with an inert liquid diluent. The tablets may be optionally coated or scored and may be formulated so as to provide controlled release (e.g. delayed, sustained, or pulsed release, or a combination of immediate release and controlled release) of the active ingredients.

Alternatively, the active ingredients may be incorporated into oral liquid preparations such as aqueous or oily suspensions, solutions, emulsions, syrups or elixirs. Formulations containing the active ingredients may also be presented as a dry product for constitution with water or another suitable vehicle before use. Such liquid preparations may contain conventional additives such as suspending agents (e.g. sorbitol syrup, methyl cellulose, glucose/sugar syrup, gelatin, hydroxymethyl cellulose, carboxymethyl cellulose, aluminium stearate gel and/or hydrogenated edible fats), emulsifying agents (e.g. lecithin, sorbitan mono-oleate and/or acacia), non-aqueous vehicles (e.g. edible oils, such as almond oil, fractionated coconut oil, oily esters, propylene glycol and/or ethyl alcohol), and preservatives (e.g. methyl or propyl p-hydroxybenzoates and/or sorbic acid).

Topical compositions, which are useful for treating disorders of the skin or of membranes accessible by digitation (such as membrane of the mouth, vagina, cervix, anus and rectum), include creams, ointments, lotions, sprays, gels and sterile aqueous solutions or suspensions. As such, topical compositions include those in which the active ingredients are dissolved or dispersed in a dermatological vehicle known in the art (e.g. aqueous or non-aqueous gels, ointments, water-in-oil or oil-in-water emulsions). Constituents of such vehicles may comprise water, aqueous buffer solutions, non-aqueous solvents (such as ethanol, isopropanol, benzyl alcohol, 2-(2-ethoxyethoxy)ethanol, propylene glycol, propylene
glycol monolaurate, glycofurol or glycerol), oils (e.g. a mineral oil such as a liquid paraffin, natural or synthetic triglycerides such as Miglyol™, or silicone oils such as dimethicone). Depending, inter alia, upon the nature of the formulation as well as its intended use and site of application, the dermatological vehicle employed may contain one or more components selected from the following list: a solubilising agent or solvent (e.g. a β-cyclodextrin, such as hydroxypropyl β-cyclodextrin, or an alcohol or polyol such as ethanol, propylene glycol or glycerol); a thickening agent (e.g. hydroxyethyl cellulose, hydroxypropyl cellulose, carboxymethyl cellulose or carbomer); a gelling agent (e.g. a polyoxyethylene-polyoxypropylene copolymer); a preservative (e.g. benzyl alcohol, benzalkonium chloride, chlorhexidine, chlorbutol, a benzoate, potassium sorbate or EDTA or salt thereof); and pH buffering agent(s) (e.g. a mixture of dihydrogen phosphate and hydrogen phosphate salts, or a mixture of citric acid and a hydrogen phosphate salt). Topical formulations may also be formulated as a transdermal patch.

Methods of producing topical pharmaceutical compositions such as creams, ointments, lotions, sprays and sterile aqueous solutions or suspensions are well known in the art. Suitable methods of preparing topical pharmaceutical compositions are described, e.g. in WO9510999, US 6974585, WO2006048747, as well as in documents cited in any of these references.

Topical pharmaceutical compositions according to the present invention may be used to treat a variety of skin or membrane disorders, such as infections of the skin or membranes (e.g. infections of nasal membranes, axilla, groin, perineum, rectum, dermatitic skin, skin ulcers, and sites of insertion of medical equipment such as i.v. needles, catheters and tracheostomy or feeding tubes) with any of the bacteria, fungi described above, (e.g. any of the Staphylococci, Streptococci, Mycobacteria or Pseudomonas organisms mentioned hereinbefore, such as S. aureus (e.g. Methicillin resistant S. aureus (MRSA))).

Particular bacterial conditions that may be treated by topical pharmaceutical compositions of the present invention also include the skin- and membrane-related conditions disclosed hereinbefore, as well as: acne vulgaris; rosacea (including erythematotelangiectatic rosacea, papulopustular rosacea, phymatous rosacea and ocular rosacea); erysipelas; erythrasma; eczema; eczema gangrenosum; impetigo; paronychia; cellulitis; folliculitis (including hot tub folliculitis); furunculosis; carbunculosis; staphylococcal scalded skin syndrome; surgical scarlet fever; streptococcal peri-anal disease; streptococcal toxic shock synдр ome; pitted keratolysis; trichomycosis axillaris; pyoderma; external canal ear infections; green nail
syndrome; spirochetes; necrotizing fasciitis; Mycobacterial skin infections (such as lupus vulgaris, scrofuloderma, wart tuberculosis, tuberculides, erythema nodosum, erythema induratum, cutaneous manifestations of tuberculoid leprosy or lepromatous leprosy, erythema nodosum leprosum, cutaneous *M. kansasii*, *M. melnoense*, *M. szulgai*, *M. simiae*, *M. gordonae*, *M. haemophilum*, *M. avium*, *M. intracellularare*, *M. chelonae* (including *M. abscessus*) or *M. fortuitum* infections, swimming pool (or fish tank) granuloma, lymphadenitis and Buruli ulcer (Bairnsdale ulcer, Searles' ulcer, Kakerifu ulcer or Toro ulcer)); as well as infected eczema, burns, abrasions and skin wounds.

Combinations for use according to the invention may be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredients. The pack may, e.g. comprise metal or plastic foil, such as a blister pack. Where the compositions are intended for administration as two separate compositions these may be presented in the form of a twin pack.

Pharmaceutical compositions may also be prescribed to the patient in "patient packs" containing the whole course of treatment in a single package, usually a blister pack. Patient packs have an advantage over traditional prescriptions, where a pharmacist divides a patients' supply of a pharmaceutical from a bulk supply, in that the patient always has access to the package insert contained in the patient pack, normally missing in traditional prescriptions. The inclusion of the package insert has been shown to improve patient compliance with the physician's instructions.

The compounds for use in the present invention are commercially available and/or may be prepared using conventional methods known in the art. Piperine may for example be extracted from black pepper using dichloromethane. This extraction is known in the art.

Suitable dosages and formulations for the administration of colistin and pharmaceutically acceptable derivatives thereof are known in the art.

A suitable dosage and formulation for colistin sulfate is for instance described in the product label for Colomycin® which can be found at [http://www.medicines.org.uk/emc/medicine/6301/SPC/Colomycin+Tablets/](http://www.medicines.org.uk/emc/medicine/6301/SPC/Colomycin+Tablets/)

A suitable dosage and formulation for colistimethate sodium is described in the product label for colistimethate sodium 1. Million I.U. Powder for Solution for Injection which can be found
at [https://www.medicines.org.uk/emc/medicine/23413](https://www.medicines.org.uk/emc/medicine/23413) Suitable dosages and formulations for the administration of piperine are also known in the art. In the U.S. for example, piperine is commercially available as Bioperine®.

The administration of the combination of the invention by means of a single patient pack, or patients packs of each composition, including a package insert directing the patient to the correct use of the invention is a desirable feature of this invention.

According to a further embodiment of the present invention there is provided a patient pack comprising at least one active ingredient of the combination according to the invention and an information insert containing directions on the use of the combination of the invention.

In another embodiment of the invention, there is provided a double pack comprising in association for separate administration, an antimicrobial agent, preferably having biological activity against clinically latent microorganisms, and one or more of the compounds disclosed herein preferably having biological activity against clinically latent microorganisms.

The amount of active ingredients required for use in treatment will vary with the nature of the condition being treated and the age and condition of the patient, and will ultimately be at the discretion of the attendant physician or veterinarian. In general however, doses employed for adult human treatment will typically be in the range of 0.02 to 5000 mg per day, preferably 1 to 1500 mg per day. The desired dose may conveniently be presented in a single dose or as divided doses administered at appropriate intervals, e.g. as two, three, four or more sub-doses per day.

**Biological Tests**

Test procedures that may be employed to determine the biological (e.g. bactericidal or antimicrobial) activity of the active ingredients include those known to persons skilled in the art for determining:

(a) bactericidal activity against clinically latent bacteria; and
(b) antimicrobial activity against log phase bacteria.

In relation to (a) above, methods for determining activity against clinically latent bacteria include a determination, under conditions known to those skilled in the art (such as those
described in *Nature Reviews, Drug Discovery* 1, 895-910 (2002), the disclosures of which are hereby incorporated by reference), of Minimum Stationary-cidal Concentration ("MSC") or Minimum Dormicidal Concentration ("MDC") for a test compound.

By way of example, WO2000028074 describes a suitable method of screening compounds to determine their ability to kill clinically latent microorganisms. A typical method may include the following steps:

1. growing a bacterial culture to stationary phase;
2. treating the stationary phase culture with one or more antimicrobial agents at a concentration and or time sufficient to kill growing bacteria, thereby selecting a phenotypically resistant sub-population;
3. incubating a sample of the phenotypically resistant subpopulation with one or more test compounds or agents; and
4. assessing any antimicrobial effects against the phenotypically resistant subpopulation.

According to this method, the phenotypically resistant sub-population may be seen as representative of clinically latent bacteria which remain metabolically active *in vivo* and which can result in relapse or onset of disease.

In relation to (b) above, methods for determining activity against log phase bacteria include a determination, under standard conditions (i.e. conditions known to those skilled in the art, such as those described in WO 2005014585, the disclosures of which document are hereby incorporated by reference), of Minimum Inhibitory Concentration ("MIC") or Minimum Bactericidal Concentration ("MBC") for a test compound. Specific examples of such methods are described below.

**Examples**

The checkerboard method used in the Examples followed the protocols detailed in Antimicrob Chemo (2013) 68, 374-384.

**Example 1: In vitro synergistic effect of piperine (HT013018) and colistin against log phase NDM-1 *Escherichia coli* using the checkerboard method**
Growth of bacteria

Log phase growth of NDM-1 *Escherichia coli* was carried out as described in the art.

Piperine and colistin were obtained from commercially available sources. Colistin was in the form of colistimethate sodium.

The effects of each combination of the present invention were examined by calculating the fractional inhibitory concentration index (FICI) of each combination, as follows:

\[
\text{FICI} = \frac{\text{MIC of drug A, tested in combination}}{\text{MIC of drug A, tested alone}} + \frac{\text{MIC of drug B, tested in combination}}{\text{MIC of drug B, tested alone}}.
\]

The interaction of the combination was defined as showing synergy if the FICI was \(\leq 0.5\), no interaction if the FICI was \(>0.5\) but \(<4.0\) and antagonism if the FICI was \(>4.0\).

### Table

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The FICI was equal to 0.5 indicating that colistin (colistimethate sodium) and piperine exhibited a synergistic effect against log phase NDM-1 *E.coli*.

**Example 2:** *In vitro* synergistic effect of colistin and piperine (HT013018) against log phase NDM-1 *Enterobacter cloacae* using the chequerboard method

Growth of bacteria

Log phase growth of NDM-1 *Enterobacter cloacae* was carried out as described in the art.
Piperine and colistin were obtained from commercially available sources. Colistin was in the form of colistimethate sodium.

The effects of each combination of the present invention were examined by calculating the fractional inhibitory concentration index (FICI) of each combination in the same manner as for Example 1.

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<th>BAA2468</th>
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</table>

The FICI was equal to 0.1875 indicating that colistin (colistimethate sodium) and piperine exhibited a synergistic effect against log phase NDM-1 Enterobacter cloacae.

Example 3: *In vitro* synergistic effect of colistin and piperine (HT013018) against log phase NDM-1 Klebsiella pneumoniae using the chequerboard method

**Growth of bacteria**
Log phase growth of NDM-1 *K.pneumoniae* was carried out as described in the art.

Piperine and colistin were obtained from commercially available sources. Colistin was in the form of colistimethate sodium.

The effects of each combination of the present invention were examined by calculating the fractional inhibitory concentration index (FICI) of each combination in the same manner as for Example 1.
The FICI was equal to 0.15625 indicating that colistin (colistimethate sodium) and piperine exhibited a synergistic effect against log phase NDM-1 *K. pneumoniae*.

**Example 4**: *In vitro* synergistic effect of piperine in combination with colistin against log phase NDM-1 *Klebsiella Pneumoniae*.

The objective of this example was to test the synergistic effect of piperine and colistin in combination against log phase NDM-1 *Klebsiella Pneumoniae* (BAA2473) by time-kill methods over a time period of 24 hours.

As described hereinabove, time-kill methods are another accepted test for ascertaining the presence or absence of synergy, and involve comparing the dynamic effect of a drug combination with each drug alone when assessing the effect on bacterial log or stationary-growth over time. The results can either show that the drug combination is synergistic, additive or antagonistic.

**Materials and Methods**

1. Bacterial strain used: BAA2473 strain of Klebsiella pneumoniae

2. Growth of bacteria: Log phase growth of BAA2473 was carried out according to known methods in the art, e.g. SOP R-005-00 Log Phase Growth of Bacteria.

3. Compound preparation:
   i. Piperine was ordered from Sigma UK.
ii. Colistin was obtained as Colistimethate sodium (Forest Laboratories UK 1 million l. U. 80 mg).

4. The overnight culture was diluted with nutrient broth (Oxoid) to 107 CFU/ml and 280 µl and 290 µl of the culture was added to each combination well and drug respectively, to make the final concentration of 300 µl.

5. Incubation of the compounds with the bacterial suspension was carried out for 24 hours. At 0, 2, 4, 7 and 24 hours, CFU counts were performed to measure the kill effects of the drug combination.

Results and discussion

The results are shown in Figures 1 to 4.

Figures 1 to 4 show that piperine at concentrations of 256, 128 and 64 mg/ml had little or no effect against NDM-1 *K. pneumoniae*. When used in combination with colistin, however, a significant synergistic effect can be seen. Figures 1 and 2 demonstrate a complete kill of bacteria at 7 hours when piperine and colistin (colistimethate sodium) are used in combination, and Figures 3 and 4 demonstrate a complete kill of bacteria after 24 hours for the combination.

In view of the lack of activity for each of the drugs alone against NDM-1 *K. pneumoniae*, the synergy seen for the combination of the claimed invention is a surprising and advantageous technical effect for the treatment of microbial infections.
Claims

1. A combination comprising piperine or a pharmaceutically acceptable derivative or stereoisomer thereof, and a polymyxin selected from polymyxin B and colistin, or a pharmaceutically acceptable derivative thereof.

2. The combination according to claim 1, wherein the polymyxin is colistin or colistimethate sodium.

3. The combination according to claim 1 or claim 2, wherein the combination comprises piperine and colistimethate sodium.

4. A combination according to any one of claims 1 to 3 for use in the treatment of a microbial infection, preferably for killing multiplying and/or clinically latent microorganisms associated with a microbial infection.

5. A combination for use according to claim 4, wherein the microbial infection is a bacterial infection, preferably a gram-negative bacterial infection.

6. A combination for use according to claim 5, wherein the bacterial infection is caused by *E. coli*, *Klebs. pneumoniae* or *Enterobacter cloacae*.

7. A combination for use according to claim 5 or claim 6, wherein the infection is caused by a drug-resistant strain of the bacteria.

8. Use of a piperine or a pharmaceutically acceptable derivative or stereoisomer thereof in combination with a polymyxin selected from polymyxin B and colistin, or a pharmaceutically acceptable derivative thereof, for the manufacture of a medicament for the treatment of a microbial infection.

9. Use according to claim 8, wherein polymyxin is colistin or colistimethate sodium.

10. Use according to claim 8 or claim 9, wherein the microbial infection is a bacterial infection.

11. Use according to claim 10, wherein the bacterial infection is a gram-negative bacterial infection, preferably caused by *E. coli*, *Klebs. pneumoniae* or *Enterobacter cloacae*.

12. Use according to claim 11, wherein the infection is caused by a drug-resistant strain.
13. Use according to any one of claims 8 to 12 for the treatment of abscesses, asthma, bacillary dysentery, bacterial conjunctivitis, bacterial keratitis, bacterial vaginosis, bone and joint infections, bronchitis (acute or chronic), brucellosis, burn wounds, cat scratch fever, cellulitis, chancroid, cholangitis, cholecystitis, cystic fibrosis, cystitis, nephritis, diffuse panbronchiolitis, dental caries, diseases of the upper respiratory tract, empyema, endocarditis, endometritis, enteric fever, enteritis, epididymitis, epiglottitis, eye infections, furuncles, gardnerella vaginitis, gastrointestinal infections (gastroenteritis), genital infections, gingivitis, gonorrhea, granuloma inguinale, Haverhill fever, infected burns, infections following dental operations, infections in the oral region, infections associated with prostheses, intraabdominal abscesses, Legionnaire's disease, leptospirosis, listeriosis, liver abscesses, Lyme disease, lymphogranuloma venerium, mastitis, mastoiditis, meningitis and infections of the nervous system, non-specific urethritis, ophthalmitis (e.g. ophthalmitis neonatorum), osteomyelitis, otitis (e.g. otitis externa and otitis media), orchitis, pancreatitis, paronychia, pelvicoperitonitis, peritonitis, peritonitis with appendicitis, pharyngitis, pleural effusion, pneumonia, postoperative wound infections, postoperative gas gangrene, prostatitis, pseudo-membranous colitis, psittacosis, pyelonephritis, Q fever, rat-bite fever, Ritter's disease, salmonellosis, salpingitis, septic arthritis, septic infections, septicaemia, systemic infections, tonsillitis, trachoma, typhoid, urethritis, urinary tract infections, wound infections; or infections with, Escherichia coli, Klebs. pneumoniae, Klebs. oxytoca, Pr. mirabilis, Pr. rettgeri, Pr. vulgaris, Haemophilis influenzae, Enterococcus faecalis, Enterococcus faecium, and Enterobacter cloacae.

14. A pharmaceutical composition comprising piperine or a pharmaceutically acceptable derivative or stereoisomer thereof, in combination with a polymyxin selected from polymyxin B and colistin, or a pharmaceutically acceptable derivative thereof and a pharmaceutically acceptable adjuvant, diluent or carrier.

15. A product comprising piperine or a pharmaceutically acceptable derivative or stereoisomer thereof, in combination with a polymyxin selected from colistin or polymyxin B, as a combined preparation for simultaneous, separate or sequential use in treating a microbial infection.
A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K31/4525 A61K38/12 A61P31/04
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, BIOSIS, CHEM ABS Data, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>page 1, paragraph 1 page 4, paragraph 1 page 6, line 19 - page 8, line 19 example 4; tables 1,7</td>
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Further documents are listed in the continuation of Box C. See patent family annex.

Date of the actual completion of the international search 7 September 2017

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

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## INTERNATIONAL SEARCH REPORT

Information on patent family members

### Patent document cited in search report

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