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(54) **Title:** FULVIC ACID AND ANTIBIOTIC COMBINATION FOR THE INHIBITION OR TREATMENT OF MULTI-DRUG RESISTANT BACTERIA

(57) **Abstract:** Fulvic acid as the active ingredient is used in the treatment or inhibition of mutti-drug resistant bacteria, in particular NDM-1 bacteria producing carbapenemase or extended- spectrum β -lactamase (ES8L) resistant bacteria. The multi-drug resistant bacteria may be gram negative bacteria including, but not limited to, Klebsiella pneumoniae or Escherichia coli.. The fuivic acid can be provided in combination with one or more antibiotics from the class of carbapenems or polymyxin antibiotics.

FULVIC ACID AND ANTIBIOTIC COMBINATION FOR THE INHIBITION OR TREATMENT OF MULTI-DRUG RESISTANT BACTERIA

BACKGROUND OF THE INVENTION

THIS invention relates to fulvic acid as the active ingredient for use in the treatment or inhibition of multi-drug resistant bacteria, in particular NDM-1 bacteria producing carbapenemase or extended-spectrum β -lactamase (ESBL) resistant bacteria. The invention further relates to fulvic acid as an active ingredient in combination with one or more antibiotics from the class of carbapenems or polymyxin antibiotics for use in the treatment of multi-drug resistant bacteria, in particular NDM-1 bacteria producing carbapenemase.

Fulvic acid is a fraction of the humic substances that are formed during the decay of plant and animal residues in the environment (MacCarthy *et al.*, 1985) and that is soluble in water under all pH conditions. It is also generally of lower molecular size and weight and lower in colour intensity than humic acids also found in humic substances.

Typically, fulvic acids have been produced from bituminous coal using a controlled wet oxidation process (Bergh *et al.*, 1997). These fulvic acids are referred to as oxifulvic acids. One such process for production of fulvic acids from coal was described in US patent no. 4,912,256.

Such fulvic acids have been used in the treatment of inflammation, acne, eczema, and bacterial, fungal and viral infections such as described in International patent publication WO00/19999. Furthermore, US patents nos. 4,999,202 and 5,204,368

disclose compositions containing fulvic acid, salt or a derivative thereof, which have bacteriostatic or bacteriocidal properties and are useful as disinfectants.

However, fulvic acids derived from oxidation of coal contain high concentrations of heavy metals including aluminium, mercury, cadmium, chromium and lead that are now known to be harmful to humans and should be avoided in pharmaceutical preparations. International patent publication WO2007/125492 discloses a fulvic acid composition derived from a carbohydrate source by a process of wet oxidation and a method of producing such a composition. As a result of the carbohydrate starting material, the fulvic acid produced from this process contains a low content of these harmful elements. Such a composition is described as being useful for pharmaceutical application.

SUMMARY OF THE INVENTION

According to one aspect of the invention, there is provided fulvic acid, or a salt, ester or derivative thereof as the active ingredient for use in the treatment or inhibition of multi-drug resistant bacteria.

In particular, the multi-drug resistant bacteria are NDM-1 positive bacteria producing carbapenemase, or extended-spectrum β -lactamase (ESBL) resistant bacteria. For example, the multi-drug resistant bacteria may be gram negative bacteria including, but not limited to, *Klebsiella pneumoniae* or *Escherichia coli*.

According to a further aspect of the invention, there is provided a combination comprising fulvic acid, or a salt, ester or derivative thereof as an active ingredient together with one or more antibiotics from the class of carbapenems or polymyxin antibiotics for use in the treatment of multi-drug resistant bacteria.

In particular, the multi-drug resistant bacteria are NDM-1 positive bacteria producing carbapenemase. For example, the multi-drug resistant bacteria may be gram negative bacteria including, but not limited to, *Klebsiella pneumoniae*.

Preferably, the carbapenem is selected from the group consisting of Meropenem, Ertapenem, Doripenem, Panipenem/Betamipron, and Biapenem.

Preferably, the polymyxin antibiotic is selected from the group consisting of colistin sulfate and colistimethate sodium (colistin methanesulfonate sodium, colistin sulfomethate sodium).

The fulvic acid, or a salt, ester or derivative thereof may have any pH, from acid to basic, although typically the pH is from acidic to neutral. The fulvic acid may be in the form of a solution buffered to a suitable pH. Preferably, the fulvic acid is either in the form of the acid or as a salt, for example a potassium salt.

The preferred fulvic acid is a carbohydrate derived fulvic acid (CHD-FA) such as that described in WO2007/125492. The fulvic acid described in this publication has a molecular weight not exceeding 20,000 Daltons, and a low content of the elements aluminium, mercury, cadmium, chromium and lead and is safe for pharmaceutical use in humans and animals. Preferably, the content of these elements does not exceed 20 ppm. The fulvic acid may be derived from a carbohydrate such as a saccharide. The preferred saccharide is sucrose, glucose or fructose.

The fulvic acid, or a salt, ester or derivative thereof or the combination may be formulated into a pharmaceutical dosage form, more particularly a liquid, tablet, capsule, cream, gel or other pharmaceutical dosage forms known to those skilled in the art.

According to a further aspect of the invention there is provided the use of the fulvic acid, or a salt, ester or derivative thereof or the combination according to the invention in the manufacture of a pharmaceutical composition for use in a method of treatment or inhibition of multi-drug resistant bacteria in a subject, the method comprising administration of the fulvic acid, or a salt, ester or derivative thereof, or the combination to the subject.

According to a further aspect of the invention there is provided a method of treating, killing, or inhibiting multi-drug resistant bacterial growth by the use of the fulvic acid, or a salt, ester or derivative thereof, or the combination of the invention.

In particular, the method of treating, killing, or inhibiting multi-drug resistant bacterial growth may be in a subject by administration of an effective amount of the fulvic acid, or a salt, ester or derivative thereof, or the combination of the invention to the subject.

The subject may be a human or non-human animal.

In particular, the multi-drug resistant bacteria are NDM-1 positive bacteria producing carbapenemase, or extended-spectrum β -lactamase (ESBL) resistant bacteria. For example, the multi-drug resistant bacteria may be gram negative bacteria including, but not limited to, *Klebsiella pneumoniae* or *Escherichia coli*.

The administration may be oral, intravenous, topical or any other suitable form of administration.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 Shows the mean thigh burden following infection with *K. pneumoniae* (ATCC BAA2146, NDM-1⁺) in all treatment groups.

Figure 2 Shows a scattergram of the thigh burden following infection with *K. pneumoniae* (ATCC BAA2146, NDM-1⁺) in all treatment groups.

DESCRIPTION OF PREFERRED EMBODIMENTS

Fulvic acid as the active ingredient for use in the treatment of multi-drug resistant bacteria, in particular NDM-1 bacteria producing carbapenemase or extended-spectrum β -lactamase (ESBL) resistant bacteria is herein described. In addition, fulvic acid as an active ingredient in combination with one or more antibiotics from the class of carbapenems or polymyxin antibiotics for use in the treatment of multi-drug resistant bacteria, in particular NDM-1 bacteria producing carbapenemase, is described.

New Delhi metallo-beta-lactamase-1 (or NDM-1) was first detected in a *Klebsiella pneumoniae* isolate in 2008. However, it was later detected in bacteria around the world, including in India, Pakistan, the United Kingdom, North America, Japan and Brazil. Generally, the enzyme is produced by Gram-negative bacteria such as *Escherichia coli*, *Acinetobacter* and *Klebsiella*, but the NDM-1 gene can spread by

horizontal gene transfer between different strains of bacteria, thereby spreading the incidence of resistant infections. Gram-negative bacteria in particular are promiscuous with their exchange of DNA fragments and so resistance such as NDM-1 can spread widely through the gram negative bacterial population.

Extended-spectrum β -lactamases (ESBLs) are beta-lactamases that hydrolyze extended-spectrum cephalosporins such as cefotaxime, ceftriaxone, ceftazidime and oxyimino-monobactam aztreonam, all with an oxyimino side chain.

NDM-1 is an enzyme that makes bacteria resistant to a broad range of beta-lactam antibiotics, such as the antibiotics of the carbapenem family. For example, carbapenems include Meropenem, Ertapenem, Doripenem, Panipenem/betamipron, and Biapenem. Unfortunately, these antibiotics are presently the mainstay for the treatment of antibiotic-resistant bacterial infections and so this resistance gene is a cause for great concern. The NDM-1 protein belongs to a large family of beta-lactamase enzymes, the carbapenemases, and infections by bacteria producing carbapenemases are very difficult to treat since there are almost no new compounds that will work against these multi-drug-resistant bacteria. Furthermore, due to the desire to avoid development of generalized resistance and the fact that, in general, carbapenems have poor oral bioavailability, they are selectively administered intravenously in the hospital setting.

An alternative antibiotic to the carbapenems that has been used successfully against multi-drug resistant carbapenemase producing bacteria is Colistin. Colistin (or polymyxin E) is an antibiotic produced by certain strains of *Bacillus polymyxa* var. *colistinus*. It is also a last-resort antibiotic for multidrug-resistant bacteria such as *Klebsiella*, *Pseudomonas aeruginosa*, and *Acinetobacter*, but unfortunately resistance to Colistin has also been identified. Colistin also is not absorbed via the gastrointestinal tract and therefore must be given intravenously. Furthermore, the dosing is complicated.

Therefore, an alternative effective agent which can be used orally would be highly advantageous.

The fulvic acid was that described in, and produced by the method described in WO 2007/125492 and is hereinafter referred to as CHD-FA.

In the first study described herein, the antibacterial efficacy of fulvic acid against a number of multi-drug resistant bacterial strains was assessed.

The second study assessed the the antibacterial efficacy of fulvic acid together with antibiotics from the carbapenem family against a NDM-1 multi-drug resistant bacterial strain.

The following examples are for the purpose of illustration only and are not to be construed as limiting on the invention in any way.

Example 1: - Antibacterial activity against multi-resistant bacteria of Fulvic Acid

Summary

The *in vitro* efficacy of CHD-FA for activity against multi-resistant Enterobacteriaceae was assessed. The CHD-FA active ingredient was stored in the dark at room temperature following delivery.

Methods

a) Strains

Susceptibility tests were performed on a range of multi-drug resistant Enterobacteriaceae strains. Details of the strains used are outlined in table 1.

b) Revival and Growth of the Strains

All strains were recovered from long-term storage at -80°C by sub-culturing onto Cled agar plates. Plates were then incubated in air at 35-37°C for 24 hours. Following

visual checks to ensure purity and appropriate colony characteristics, isolates were deemed suitable for use.

Table 1 : NDM-1 and KPC containing Enterobacteriaceae strains (study 1)

SPECIES	Number	STRAIN	COMMENTS
<i>Klebsiella pneumoniae</i>	ATCC BAA 2146	New Delhi metallo-beta-lactamase (NDM-1) positive	<i>bla</i> _{KPC} -ve by PCR <i>bla</i> _{NDM} +ve by PCR
<i>Klebsiella pneumoniae</i>	NCTC1 3443	New Delhi metallo-beta-lactamase (NDM-1) positive	N/A
<i>Klebsiella pneumoniae</i>	ATCC BAA 1705	Modified Hodge Test (MHT) positive control designation	Strain produces <i>K. pneumoniae</i> carbapenemase (KPC) <i>bla</i> _{KPC} +ve by PCR
<i>Klebsiella pneumoniae</i>	ATCC 700603	ESBL control strain	produces beta-lactamase SHV-18
<i>Escherichia coli</i>	Clinical 7	Clinical strain	ESBL Multi resistant strain
<i>Escherichia coli</i>	ATCC 25922	CLSI control strain	Susceptible isolate

c) Preparation of the Inoculum

The inocula for each strain were prepared by picking 5-10 distinct colonies from the culture plates and suspending them in 3 ml of sterile saline. The inoculum was resuspended by vigorous shaking on a vortex mixer for 15 seconds. The turbidity was then adjusted to McFarland standard 0.5 ($1-5 \times 10^8$ CFU/mL). The inoculum was further diluted in Mueller Hinton broth for MIC tests to give a final inoculum in each well of $2-8 \times 10^5$ CFU/ml.

d) MIC Assay Conditions

MICs were tested in Mueller Hinton broth in accordance with the appropriate CLSI guidelines.

STEP 1: Addition of Test Article

- a. The stock solution was provided as a 4% stock solution. This was further diluted in the appropriate media (Mueller Hinton broth) to give a top starting concentration of 2% in the assay. In addition, for each strain, a comparator control was included. The final concentration range for the comparator control (ciprofloxacin) was 0.03 -16 mg/L. 100 μ L of Mueller Hinton broth was dispensed into each well in columns 2-12. 200 μ L of the appropriate test compound solution (at 4%) was dispensed into each well in column 1.
- b. 100 μ L aliquots were pipetted from column 1 wells and dispensed into column 2 with a multichannel pipette (\pm 2% coefficient of variation) thus diluting two-fold. 100 μ L samples were then pipetted from column 2 wells and dispensed into column 3. The process was repeated through to column 10. The final 100 μ L of diluted drug from column 10 was then discarded. Row 11 acted as a positive control (no drug or test article, organisms added), Row 12 acted as a negative control (no drug or test article, and no organisms added).

STEP 2: Addition of Bacterial Strains

100 μ L of the appropriate inoculum suspension in Mueller Hinton broth was added to the appropriate wells. This resulted in a well containing 200 μ L final volume (made up of 100 μ L diluted compound or diluents and 100 μ L of inoculum or broth alone).

STEP 3: Incubation of Assay Plates

All plates were incubated in the dark at 35-37°C in air for 18-24 hours.

STEP 4: Reading of Plates

Plates were read visually and spectrophotometrically (450nm) where possible, 20 or 72 hours post inoculation. Endpoints of 50%>, 80%> and 100%> inhibition were determined (or CLSI interpretation endpoints following visual examination).

Results**a) Visual MIC values (50%, 80% and 100% inhibition)**

The CHD-FA alone was effective against NDM-1, KPC and ESBL positive *K. pneumonia* and multi-drug resistant *E. coli* strains with a 100% MIC value of only 0.06 – 0.12% CHD-FA as shown in Table 2.

This compares favourably against ciprofloxacin which was not effective against either NDM-1, nor KPC positive *K. pneumonia* strains, having an MIC of more than 16 µg/ml. CHD-FA alone was as effective as ciprofloxacin against *K. pneumonia* and *E. coli* ESBL positive strains.

Table 2: MIC efficacy data of CHD-FA against various Enterobacteriaceae strains

Compound	<i>K. pneumoniae</i> ATCC BAA 2146			<i>K. pneumoniae</i> NCTC 13443			<i>K. pneumoniae</i> ATCC BAA 1705			<i>K. pneumoniae</i> ATCC700603			<i>E. coli</i> Clinical strain 7			<i>E. coli</i> ATCC 25922		
	100%	80%	50%	100%	80%	50%	100%	80%	50%	100%	80%	50%	100%	80%	50%	100%	80%	50%
CHD-FA (%)	0.12	0.12	0.12	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06
Ciprofloxacin (µg/ml)	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16	0.25	0.25	0.25	0.06	0.06	0.06

Summary

CHD-FA was highly effective against multi-drug resistant Gram negative bacilli including NDM-1 positive strains with all organisms being 100% inhibited using a concentration of $\leq 0.12\%$ CHD-FA.

Example 2 – Efficacy of CHD-FA alone or in combination with a carbapenem antibiotic in a mouse Thigh Model of *K. pneumonia* Infection

Methods

a) Regulatory

All animal experiments were performed under UK Home Office Licence with local ethical committee clearance. All experiments were performed by technicians that have completed parts 1, 2 and 3 of the Home Office Personal License course and hold current personal licenses.

b) Mouse Strain

Mice used in this study were supplied by Charles River UK and were specific pathogen free. The strain of mouse used was CD1, which is a well characterized outbred strain. Mice were 16-18 g on receipt at Euprotec's facility and were allowed to acclimatize for 7 days (target weight at the start of the experiment 22-25 g).

c) Animal Housing

Mice were housed in sterile individual ventilated cages exposing mice at all times to HEPA filtered sterile air (<http://www.tecniplast.it/assets/panorama/Panorama30.pdf>). Mice had free access to food and water (sterile) and had sterile aspen chip bedding (changed every 3-4 days or as appropriate). The room temperature was $22^{\circ}\text{C} \pm 1^{\circ}\text{C}$,

with a relative humidity of 50-60% and maximum background noise of 56 dB. Mice were exposed to 12 hour light/dark cycles with dawn/dusk phases.

d) *Klebsiella pneumoniae* Isolate

Klebsiella pneumoniae (ATCC BAA2146 NDM-1⁺ (bla_{ndm})) was used throughout the study.

Experimental Design

a) Experimental Group Size

In this study, 6 mice were used in each treatment group.

b) Immunosuppression

Mice were rendered temporarily neutropenic by immunosuppression with cyclophosphamide at 150 mg/kg four days before infection and 100 mg/kg one day before infection by intraperitoneal injection. The immunosuppression regime leads to neutropenia starting 24 hours post administration which continues throughout the study.

c) Infection

24 hours post the second round of immunosuppression mice were infected with *K. pneumoniae* intramuscularly into both lateral thigh muscles using 2.5x10⁸ cfu/mouse thigh.

d) Antibacterial Therapy

Antibacterial treatment was initiated 2 hours post infection (Table 3) and was administered twice (exactly 12 hours apart). An aliquot of 4% CHD-FA stock solution as adjusted to pH 5 with 10 M sodium hydroxide solution then diluted either 1:2 or 1:8

with 0.9% saline to give a dosing solution of 2% (200 mg/kg) and 0.5% (50 mg/kg) CHD-FA respectively.

Colistin was diluted in 0.9% saline to give a dosing solution of 4 mg/ml (40 mg/kg). Meropenem was diluted in 0.9% saline to give a dosing solution of 3 mg/ml (30 mg/kg). The vehicle was 0.9% saline. Following reconstitution, the suspensions were vortexed to ensure complete dissolution.

CHD-FA was administered by gavage, whereas Colistin, Meropenem and the vehicle were administered IV at 10 ml/kg (requiring individual mice to be administered ~0.25 ml/dose).

e) Endpoints

24 hours post infection, the clinical condition of all animals was assessed prior to them being humanely euthanized. Animal weight was determined before both thighs were removed and weighed individually. Individual thigh tissue samples were homogenized in ice cold sterile phosphate buffered saline. Thigh homogenates were then quantitatively cultured on to CLED agar and incubated at 37°C for 24 hrs and colonies counted daily.

f) Statistical Analysis

Quantitative thigh tissue burden data for CHD-FA were analysed using the statistical test Kruskal Wallis test (corrected for multiple comparisons) and compared to Colistin, Meropenem and vehicle monotherapy, plus Colistin/CHD-FA and Meropenem/CHD-FA combinations.

Table 3: Experimental Treatment Groups

Box number	Treatment	Dose	Frequency	1 st Dose	2 nd Dose	Route
1	CHD-FA	0.5%	2 X daily	2h	14h	Oral
2	CHD-FA	2%	2 X daily	2h	14h	Oral
3	Colistin	40mg/kg	2 X daily	2h	14h	IV
4	Colistin + CHD-FA	40mg/kg + 0.5%	2 X daily	2h	14h	IV/Oral
5	Colistin + CHD-FA	40mg/kg + 2%	2 X daily	2h	14h	IV/Oral
6	Meropenem	30mg/kg	2 X daily	2h	14h	IV
7	Meropenem + CHD-FA	30mg/kg + 0.5%	2 X daily	2h	14h	IV/Oral
8	Meropenem + CHD-FA	30mg/kg + 2%	2 X daily	2h	14h	IV/Oral
9	Vehicle	-	2 X daily	2h	14h	IV

Results

a) *In vivo* Efficacy

A severe model of *K. pneumoniae* thigh infection was successfully established with ~11 log₁₀ cfu/g in thighs of vehicle control mice 24 hrs post infection. Moderate signs of infection were observed in vehicle treated mice. Treatment with all test articles were well tolerated during the study with no adverse effects observed.

Quantitative thigh tissue burden data indicated that *all* treatment groups demonstrated statistically significant reduction in thigh burdens compared with vehicle only treated mice (1.48 - 3.3 log₁₀ reduction, P<0.0001 for all treatment groups except Colistin 40mg/kg + CHD-FA 2%, P=0.0102 (StatsDirect - Kruskal-Wallis: all pairwise comparisons (Conover-Inman)).

Remarkably CHD-FA monotherapy administered at 2% and 0.5% (200 and 50 mg/kg) was as effective as 40 mg/kg Colistin or 30 mg/kg Meropenem at reducing the tissue burden.

The greatest reduction in burden was observed with the combination treatment group Meropenem 30mg/kg + CHD-FA 0.5%, IV+PO BD (3.3 log₁₀ reduction, P<0.0001).

The reduction in burden following CHD-FA treatment at 0.5% and 2% PO BD was not statistically different to that observed with either Colistin 40 mg/kg IV BD or Meropenem 30 mg/kg IV BD monotherapy. However, data demonstrates significantly improved efficacy when CHD-FA 5% is administered in combination with Meropenem, rather than alone (P=0.0008).

The rank efficacy order of efficacy compared with vehicle control group was as follows:

1. Meropenem 30 mg/kg + CHD-FA 0.5%, IV+PO BD (3.3 log₁₀ reduction, P<0.0001),
2. CHD-FA 2%, PO BD (2.31 log₁₀ reduction, P<0.0001),
3. CHD-FA 0.5%, PO BD (2.28 log₁₀ reduction, P<0.0001),
4. Colistin 40 mg/kg, IV BD (2.27 log₁₀ reduction, P<0.0001),
5. Colistin 40 mg/kg + CHD-FA 0.5%, IV+PO BD (2.21 log₁₀ reduction, P<0.0001),
6. Meropenem 30 mg/kg + CHD-FA2%, IV+PO BD (2.20 log₁₀ reduction, P<0.0001),
7. Colistin 40 mg/kg + CHD-FA 2%, IV+PO BD (2.20 log₁₀ reduction, P<0.0102), and
8. Meropenem 30 mg/kg, IV BD (1.74 log₁₀ reduction, P<0.0001).

Table 4 sets out a summary of the thigh tissue burden in the test mice with and without treatment.

Table 4. Thigh tissue burden summary table.

Group	Treatment	Group Average (cfu/g)	Group Ave. Log 10 cfu/g	Log reduction from vehicle	n#	No. Sterilised*
1	CHD-FA 0.5%, oral 2X daily	1358950384	9.13	2.28	12	0
2	CHD-FA 2%, oral 2X daily	1272314925	9.10	2.31	12	0
3	Colistin 40mg/kg, IV 2X daily	1388363808	9.14	2.27	12	0
4	Colistin 40mg/kg + CHD-FA 0.5%, IV+ oral 2X daily	1570868780	9.20	2.21	12	0
5	Colistin 40mg/kg + CHD-FA 2%, IV+ oral 2X daily	8468797243	9.93	1.48	12	0
6	Meropenem 30mg/kg, IV 2X daily	4729505542	9.67	1.74	12	0
7	Meropenem 30mg/kg + CHD-FA 0.5%, IV+ oral 2X daily	129247657	8.11	3.30	12	0
8	Meropenem 30mg/kg + CHD-FA 2%, IV+ oral 2X daily	1624414808	9.21	2.20	12	0
9	Vehicle	257564729875	11.41	0.00	12	0

*5 or less colonies detected on neat plate

Table 5. Kruskal-Wallis statistical tests (corrected for multiple comparisons) of test articles CHD-FA, Colistin and Meropenem in temporarily neutropenic mice infected with *Klebsiella pneumoniae* (ATCC BAA2146 (NDM-1⁺) intramuscularly.

	CHD-FA 0.5%, PO BD	CHD-FA 2%, PO BD	Colistin 40mg/kg, IV BD	Colistin 40mg/kg + CHD-FA 0.5%, IV+PO BD	Colistin 40mg/kg + CHD-FA 2%, IV+PO BD	Meropenem 30mg/kg, IV BD	Meropenem 30mg/kg + CHD-FA 0.5%, IV+PO BD	Meropenem 30mg/kg + CHD-FA 2%, IV+PO BD	Vehicle
CHD-FA 0.5%, PO BD		NS	NS	NS	P = 0.0017	NS	P = 0.0008	NS	P < 0.0001
CHD-FA 2%, PO BD			NS	NS	P = 0.0012	NS	P = 0.0011	NS	P < 0.0001
Colistin 40mg/kg, IV BD				NS	P = 0.0008	NS	P = 0.0018	NS	P < 0.0001
Colistin 40mg/kg + CHD-FA 0.5%, IV+PO BD					P = 0.0011	NS	P = 0.0013	NS	P < 0.0001
Colistin 40mg/kg + CHD-FA 2%, IV+PO BD						NS	P < 0.0001	p = 0.0044	P = 0.0102
Meropenem 30mg/kg, IV BD							P < 0.0001	NS	P < 0.0001
Meropenem 30mg/kg + CHD-FA 0.5%, IV+PO BD								P = 0.0003	P < 0.0001
Meropenem 30mg/kg + CHD-FA 2%, IV+PO BD									P < 0.0001
Vehicle									

NS: Not statistically significant

Figure 1 sets out the mean thigh burden following infection with *K. pneumonia* (ATCC BAA2146, NDM-1⁺) and all treatment groups. Figure 2 sets out a scattergram of the thigh burden following infection with *K. pneumonia* (ATCC BAA2146, NDM-1⁺) and all treatment groups. The geometric mean burden of each treatment is indicated by a horizontal bar. Above each column the log₁₀ reduction of that treatment group compared to the vehicle group is recorded.

Summary

The aim of the study was to assess the efficacy of CHD-FA administered as a monotherapy or as combination therapy with either Colistin or Meropenem in a neutropenic murine thigh infection model caused by a multi-drug resistant strain of *Klebsiella pneumoniae* (New Delhi metallo-beta-lactamase or NDM-1 positive). Efficacy was compared using tissue burdens from the thigh at the end of the experiment. Treatment was initiated 2 hours post-infection, and administered twice exactly 12 hours apart. Mice were euthanized 24 hours post infection.

A severe infection caused by *K. pneumoniae* was established in the thighs of vehicle treated mice 24 hrs post infection. However, despite this high level of infection no mice succumbed to infection prior to the end of the study, although moderate signs of infection (local tenderness) were observed.

Quantitative thigh tissue burden data indicated that all treatment groups demonstrated highly statistically significant reduction in thigh burdens compared with vehicle only treated mice.

Remarkably CHD-FA monotherapy administered at 2% and 0.5% (0.2 and 0.05 mg/kg) was as effective as 40 mg/kg Colistin or 30 mg/kg Meropenem at reducing the tissue burden.

The greatest reduction in thigh burden was observed following treatment with the combination of Meropenem 30mg/kg + CHD-FA 0.5% highlighting the fact that not only

was CHD-FA effective as monotherapy but it also augmented the treatment efficacy of the currently used drugs in a synergistic manner.

Conclusion

CHD-FA administered either as a monotherapy or as a combination therapy with either Colistin or Meropenem exhibited highly significant efficacy against multi-drug resistant *Klebsiella pneumoniae* in a localised thigh infection model. Combination therapy with 30 mg/kg Meropenem plus 50 mg/kg CHD-FA showed superior efficacy compared to the same dose of CHD-FA, Meropenem or Colistin administered as a monotherapy.

References:

Bergh J.J., Cronje I.J., Dekker J., Dekker T.G., Gerritsma L.M. & Mienie L.J. 1997. Non-catalytic oxidation of water-slurried coal with oxygen: identification of fulvic acids and acute toxicity. *Fuel* 76, 149-154 (1997).

MacCarthy P, Clapp CE, Malcolm RL, Bloom PR. Humic substances in soil and crop sciences: selected readings. Proceedings of a symposium by International Humic Substances Society, Soil Science Society of America, American Society of Agronomy and Crop Science Society of America, Chigaco, Illinois, 2 December 1985.

CLAIMS

1. Fulvic acid, or a salt thereof, as the active ingredient for use in the treatment or inhibition of multi-drug resistant bacteria.
2. Fulvic acid, or a salt thereof, according to claim 1, wherein the multi-drug resistant bacteria are NDM-1 positive bacteria producing carbapenemase, or extended-spectrum β -lactamase (ESBL) resistant bacteria.
3. Fulvic acid, or a salt thereof, according to claim 1 or claim 2, wherein the multi-drug resistant bacteria are gram negative bacteria including, but not limited to, *Klebsiella pneumoniae* or *Escherichia coli*.
4. A combination comprising fulvic acid, or a salt thereof, as an active ingredient together with one or more antibiotics from the class of carbapenems or polymyxin antibiotics for use in the treatment of multi-drug resistant bacteria.
5. Fulvic acid, or a salt thereof, according to any one of claims 1 to 3, or a combination according to claim 4, wherein the multi-drug resistant bacteria are NDM-1 positive bacteria producing carbapenemase.
6. Fulvic acid, or a salt thereof, or a combination according to claim 5, wherein the NDM-1 positive bacteria producing carbapenemase are gram negative bacteria including, but not limited to, *Klebsiella pneumonia*.
7. A combination according to claim 4, wherein the carbapenem is selected from the group consisting of Meropenem, Ertapenem, Doripenem, Panipenem/Betamipron, and Biapenem and where the polymyxin antibiotic is selected from the group consisting of colistin sulfate and colistimethate sodium (including colistin methanesulfonate sodium and colistin sulfomethate sodium).
8. Fulvic acid, or a salt thereof, according to any one of claims 1 to 3, 5 or 6, or a combination according to any one of claims 4 to 7, wherein the fulvic acid, or a salt thereof has a pH from acidic to neutral.

9. Fulvic acid, or a salt thereof, according to any one of claims 1 to 3, 5, 6 or 8, or a combination according to any one of claims 4 to 8, wherein the fulvic acid is a carbohydrate derived fulvic acid (CHD-FA).
10. Fulvic acid, or a salt thereof, according to any one of claims 1 to 3, 5, 6, 8 or 9, or a combination according to any one of claims 4 to 9, formulated into a pharmaceutical dosage form.
11. Fulvic acid, or a salt thereof, or a combination according to claim 10, wherein the pharmaceutical dosage form is a liquid, tablet, capsule, cream, gel or other pharmaceutical dosage forms known to those skilled in the art.
12. Use of fulvic acid, or a salt thereof, according to any one of claims 1 to 3, 5, 6 or 8 to 11, or a combination according to any one of claims 4 to 11 in the manufacture of a pharmaceutical composition for use in a method of treatment or inhibition of multi-drug resistant bacteria in a subject, the method comprising administration of the fulvic acid, or a salt thereof, or the combination to the subject.
13. A method of treating, killing, or inhibiting multi-drug resistant bacterial growth by the use of fulvic acid, or a salt thereof, according to any one of claims 1 to 3, 5, 6 or 8 to 11, or a combination according to any one of claims 4 to 11.
14. A method according to claim 13, wherein the treating, killing, or inhibiting multi-drug resistant bacterial growth is in a subject by administration of an effective amount of the fulvic acid, or a salt thereof, or the combination.
15. A use according to claim 12 or a method according to claim 14, wherein the subject is a human or non-human animal.
16. A use according to claim 12 or a method according to claim 14 or claim 15, wherein the multi-drug resistant bacteria are NDM-1 positive bacteria producing carbapenemase, or extended-spectrum β -lactamase (ESBL) resistant bacteria.
17. A use or method according to claim 16, wherein the multi-drug resistant bacteria are gram negative bacteria including, but not limited to, *Klebsiella pneumoniae* or *Escherichia coli*.

18. A use according to claim 12 or a method according to claim 14 or claim 15, wherein the administration is oral, intravenous, topical or any other suitable form of administration.

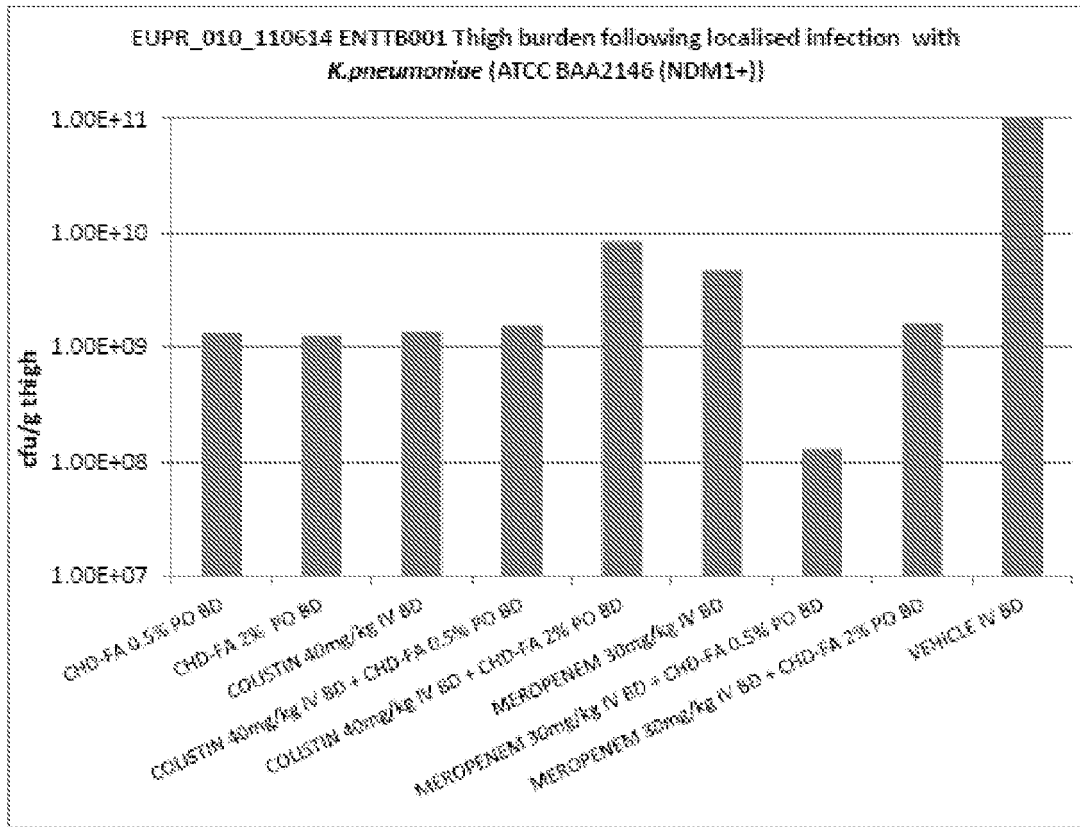


Figure 1

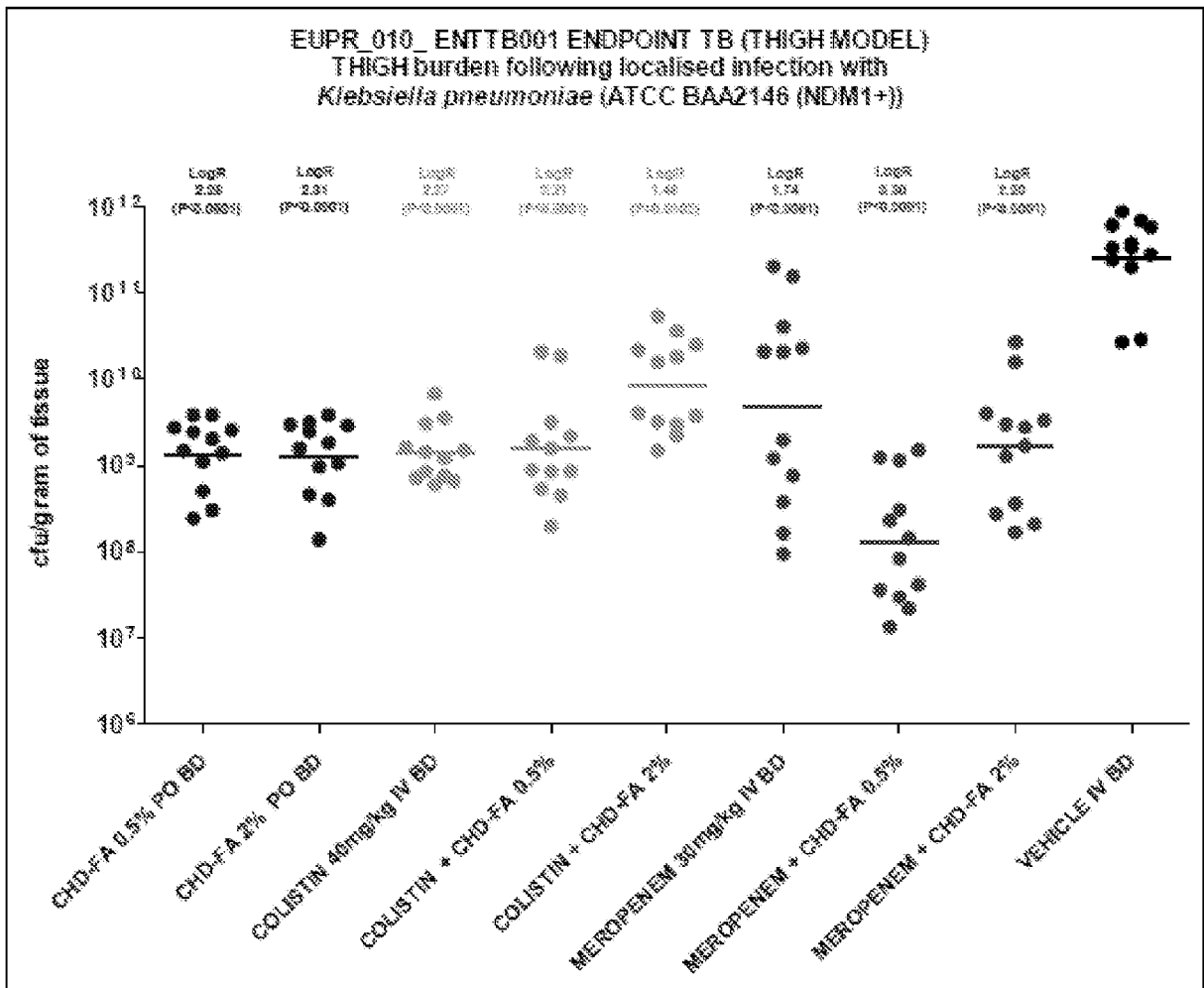


Figure 2

INTERNATIONAL SEARCH REPORT

International application No PCT/IB2013/051772

A. CLASSIFICATION OF SUBJECT MATTER INV. A61K31/352 A61K45/06 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				
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.		
X	WO 2009/147635 A1 (PFEINSMITH S A PTY LTD [ZA]; FERNANDES ANTONIO CELESTINO [ZA]; MEDLEN) 10 December 2009 (2009-12-10) pages 3,5; claim 10	1,8-15, 18		
Y	-----	1-18		
X	Anonymous: "Producers of medicinal grade heavy metal free fulvic acid", FulHold: Efficacy - Anti-Bacterial	1-3,5,6, 8-18		
Y	24 July 2011 (2011-07-24), XP002697251, Retrieved from the Internet: URL:http://web.archive.org/web/20110724004303/http://www.fulhold.com/index.php?id=96 [retrieved on 2013-05-17] the whole document	1-18		
-/--				
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input checked="" type="checkbox"/> See patent family annex.				
* Special categories of cited documents : <table style="width: 100%; border: none;"> <tr> <td style="width: 50%; border: none; vertical-align: top;"> "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 </td> <td style="width: 50%; border: none; vertical-align: top;"> "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 </td> </tr> </table>			"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
"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	Date of mailing of the international search report			
17 May 2013	19/06/2013			
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 Steendijk, Martin			

INTERNATIONAL SEARCH REPORT

International application No PCT/IB2013/051772

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	<p>-& Sandra Howsley: "Determination of the invitro efficacy of CHD-FA against multi-resistant enterobacteriaceae and mycobacteria", FulHold: FINAL REPORT - EUPR_010_101101 v1</p> <p>24 July 2011 (2011-07-24), XP002697254, Retrieved from the Internet: URL:http://web.archive.org/liveweb/http://www.fulhold.com/pics/Multi_Resistant_Enterobacteriaceae_and_Mycobacteria.pdf [retrieved on 2013-05-17] the whole document</p> <p style="text-align: center;">-----</p>	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IB2013/051772

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2009147635	A1	CN 102099027 A	15-06-2011
		EP 2317998 A1	11-05-2011
		RU 2010154511 A	20-07-2012
		US 2011207687 A1	25-08-2011
		WO 2009147635 A1	10-12-2009
