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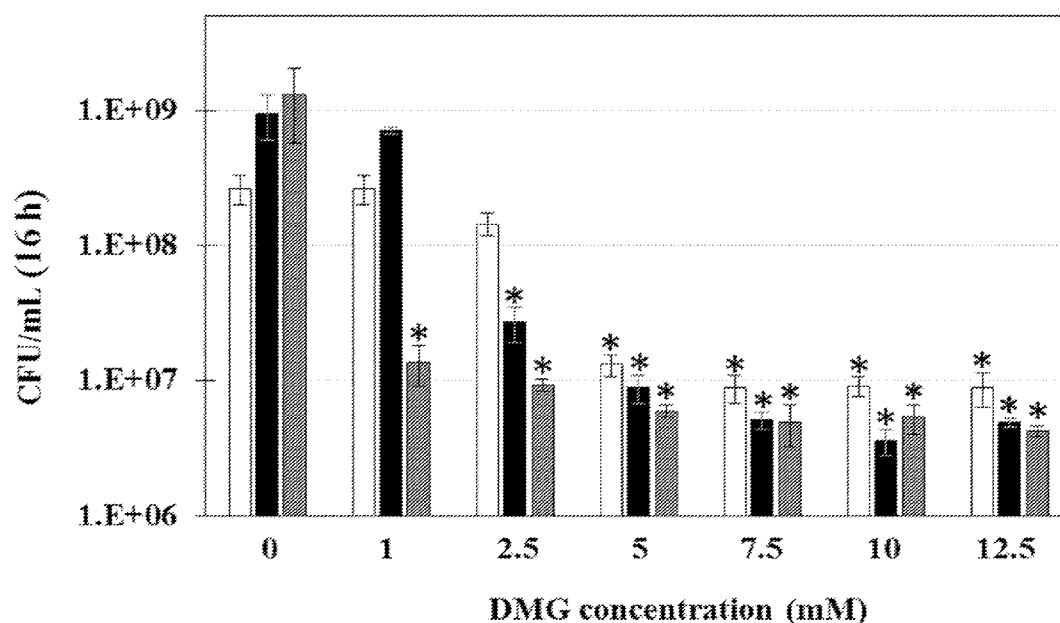
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FIG. 2



(57) Abstract: This disclosure describes compositions including dimethylglyoxime (DMG) and methods of using those compositions including, for example, to reduce the availability of nickel in the subject. In some aspects, the composition may be administered to a subject suffering from or susceptible to a bacterial infection, to a subject suffering from or susceptible to a β -amyloid peptide aggregation, to a subject suffering from or susceptible to a nickel allergy, to an obese subject, or to a subject to alter the balance of bacteria in the subject's microbiome. In some aspects, this disclosure describes using DMG or a composition including DMG to disrupt a biofilm or prevent biofilm formation.



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NICKEL CHELATION THERAPY

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CONTINUING APPLICATION DATA

This application claims the benefit of U.S. Provisional Application Serial No. 62/840,543, filed April 30, 2019, which is incorporated by reference herein.

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GOVERNMENT FUNDING

This invention was made with government support under AI121181 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND

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Enterobacteriaceae illnesses, including those caused by *Escherichia*, *Klebsiella*, *Salmonella*, *Shigella*, and *Yersinia* species, cost billions of dollars in diarrheal illness treatment and lead to millions of human deaths every year. For instance, in 2013, the annual cost associated with non-typhoidal *Salmonella* infections alone was estimated at 3.67 billion dollars in the United States. Among Enterobacteriaceae, multi-drug resistant (MDR) species pose one of the biggest public health challenges of our time. A recent study conducted over three years in a French hospital found that bloodstream infections with MDR Enterobacteriaceae accounted for more than 70 percent (%) of all bloodstream infections with MDR bacterial strains. MDR bacterial strains include, for example, extended-spectrum β -lactamase (ESBL)-producing and carbapenem-resistant Enterobacteriaceae (CRE). Resistance to drugs can emerge rapidly, and responses to these emerging public threats are slow or even nonexistent. New avenues to disable these and related pathogens would be advantageous.

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SUMMARY OF THE INVENTION

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This disclosure describes compositions including dimethylglyoxime (DMG) and methods of using those compositions including, for example, to treat infections with MDR bacterial strains.

In one aspect, this disclosure describes a method that includes administering a compound to a subject to reduce the availability of nickel in the subject, wherein the compound includes dimethylglyoxime (DMG).

In another aspect, this disclosure describes a method of treating or preventing a bacterial infection in a subject, the method comprising administering DMG to the subject.

In a further aspect, this disclosure describes a method of treating or preventing nickel allergy in a subject, a method of treating or preventing obesity in a subject, or a method of altering the balance of bacteria in the subject's microbiome, wherein the method includes administering DMG to the subject. In some embodiments, the DMG includes soluble DMG.

In yet another aspect, this disclosure describes a method of disrupting a biofilm or preventing biofilm formation, the method comprising treating a surface with DMG.

In an additional aspect, this disclosure describes a pharmaceutical composition including a chelator, the chelator including soluble DMG, wherein the pharmaceutical composition is formulated for oral or intravenous administration.

The words "preferred" and "preferably" refer to embodiments of the invention that may afford certain benefits, under certain circumstances. However, other embodiments may also be preferred, under the same or other circumstances. Furthermore, the recitation of one or more preferred embodiments does not imply that other embodiments are not useful and is not intended to exclude other embodiments from the scope of the invention.

The terms "comprises" and variations thereof do not have a limiting meaning where these terms appear in the description and claims. Such terms will be understood to imply the inclusion of a stated step or element or group of steps or elements but not the exclusion of any other step or element or group of steps or elements.

By "consisting of" is meant including, and limited to, whatever follows the phrase "consisting of." Thus, the phrase "consisting of" indicates that the listed elements are required or mandatory, and that no other elements may be present. By "consisting essentially of" is meant including any elements listed after the phrase, and limited to other elements that do not interfere with or contribute to the activity or action specified in the disclosure for the listed elements. Thus, the phrase "consisting essentially of" indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present depending upon whether or not they materially affect the activity or action of the listed elements.

Unless otherwise specified, "a," "an," "the," and "at least one" are used interchangeably and mean one or more than one.

Also herein, the recitations of numerical ranges by endpoints include all numbers subsumed within that range (for example, 1 to 5 includes 1, 1.5, 2, 2.75, 3, 3.80, 4, 5, etc.).

For any method disclosed herein that includes discrete steps, the steps may be conducted in any feasible order. And, as appropriate, any combination of two or more steps may be conducted simultaneously.

5 All headings are for the convenience of the reader and should not be used to limit the meaning of the text that follows the heading, unless so specified.

Reference throughout this specification to “one embodiment,” “an embodiment,” “certain embodiments,” or “some embodiments,” etc., means that a particular feature, configuration, composition, or characteristic described in connection with the embodiment is included in at least one embodiment of the disclosure. Thus, the appearances of such phrases in various places
10 throughout this specification are not necessarily referring to the same embodiment of the disclosure. Furthermore, the particular features, configurations, compositions, or characteristics may be combined in any suitable manner in one or more embodiments.

Unless otherwise indicated, all numbers expressing quantities of components, molecular weights, and so forth used in the specification and claims are to be understood as being modified in
15 all instances by the term “about.” As used herein in connection with a measured quantity, the term “about” refers to that variation in the measured quantity as would be expected by the skilled artisan making the measurement and exercising a level of care commensurate with the objective of the measurement and the precision of the measuring equipment used. Accordingly, unless otherwise indicated to the contrary, the numerical parameters set forth in the specification and claims are
20 approximations that may vary depending upon the desired properties sought to be obtained by the present invention. At the very least, and not as an attempt to limit the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques.

Notwithstanding that the numerical ranges and parameters setting forth the broad scope of
25 the invention are approximations, the numerical values set forth in the specific examples are reported as precisely as possible. All numerical values, however, inherently contain a range necessarily resulting from the standard deviation found in their respective testing measurements.

The above summary of the present invention is not intended to describe each disclosed embodiment or every implementation of the present invention. The description that follows more
30 particularly exemplifies illustrative embodiments. In several places throughout the application, guidance is provided through lists of examples, which examples can be used in various

combinations. In each instance, the recited list serves only as a representative group and should not be interpreted as an exclusive list.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1A shows the structure of dimethylglyoxime (DMG) and DMG-Ni. Two molecules of DMG are needed to coordinate one molecule of Ni²⁺. FIG. 1B shows disodium salt octahydrate DMG.

FIG. 2 shows the effect of DMG on the growth of multi-drug resistant (MDR) *K. pneumoniae* and *S. Typhimurium* strains. *K. pneumoniae* BAA2472 (white bars), *S. Typhimurium* 700408 (black bars), and *S. Typhimurium* 14028 (grey bars) were inoculated (approximately 5 x 10⁶ CFU/mL) in appropriate media containing increasing concentrations of DMG, as indicated, and grown at 37°C under aerobic conditions and constant shaking. Cell growth (CFU/mL) at 16 hours was determined by serial dilution and plating. Results shown represent means and standard deviations from three biological replicates. An asterisk above a bar indicates the bacterial growth (CFU/mL) was significantly lower compared to the no (0) DMG control ($P < 0.01$, Student's *t*-test).

FIG. 3A – FIG. 3B show regions of 800 MHz proton-carbon correlated spectra showing diagnostic DMG signals in liver extracts from DMG-fed mice (FIG. 3A) and no-DMG control mice (FIG. 3B). FIG. 3A. One-bond correlated methyl protons and methyl carbon signal in HSQC spectrum (top panel) and two-bond correlated methyl protons to oxime carbon signal (bottom panel). FIG. 3B. HSQC spectrum corresponding to the top panel of FIG. 3A.

FIG. 4A – FIG. 4B show DMG-chelation attenuates *S. Typhimurium* 14028 virulence in mice. Mouse survival following infection with *S. Typhimurium* 14028 and treatment with DMG (white circles) or no DMG treatment (black circles). For DMG-treated mice, a dose of 3 mg DMG (in water) was orally given 6 hours after infection with *S. Typhimurium* and then once daily, until day 7 (FIG. 4A) or until day 9 (FIG. 4B) post-inoculation. The last day of DMG treatment is indicated by an arrow, and the number of mice (n) used for each experiment is shown in the upper right box.

FIG. 5 shows DMG treatment decreases *S. Typhimurium* organ burden in mice. Organ colonization of *S. Typhimurium* strain 14028 in the livers (circles) and spleens (diamonds) of infected mice (72 hours post *S. Typhimurium* inoculation), after treatment with DMG (white symbols) or no DMG treatment (black symbols). Each symbol represents the mean (Log₁₀) CFU/mL for one organ (liver or spleen, as indicated) and each horizontal bar represents the

geometric mean of the colonization load for each group. The organ burden (mean colonization) in the DMG-treated group is significantly lower compared to the control group (no DMG), $P < 0.01$ for livers and $P < 0.025$ for spleens, respectively.

FIG. 6 shows DMG-treatment of MDR *K. pneumoniae* and MDR *S. Typhimurium* attenuates virulence in the *Galleria mellonella* insect model. *G. mellonella* larvae (n=10 for each condition) were inoculated with 5 μ L of the following: 0.8% NaCl (control), white squares; 250 mM DMG, black diamonds; 5×10^5 CFUs *K. pneumoniae* BAA2472, black circles; 250 mM DMG (left proleg) and 5×10^5 CFUs *K. pneumoniae* BAA2472 (right proleg), white circles; 5×10^5 CFUs *S. Typhimurium* 14028, gray triangles; 250 mM DMG (left proleg) and 5×10^5 CFUs *S. Typhimurium* 14028 (right proleg), white triangles.

FIG. 7 shows DMG inhibits β -amyloid peptide aggregation, as described in Example 2A.

FIG. 8 shows Zn, Ni, Mn, Cu, Co, and Fe enhance β -amyloid peptide aggregation, as described in Example 2B. In the absence of added metal, DMG was observed to slow down (100 mM DMG) or inhibit (1 mM DMG) β -amyloid peptide aggregation. When nickel was added, inhibition was observed at a 10:1 DMG:nickel ratio.

FIG. 9 shows DMG partially inhibits copper- and nickel-mediated β -amyloid peptide aggregation at a 10:1 DMG:metal ratio, as described in Example 2C.

FIG. 10 shows DMG inhibits exemplary biofilms. *H. pylori* 43504, *S. Typhimurium* 700408, and *K. pneumoniae* BAA2472 cells were incubated with DMG in 96 well plates for 48 hours (*H. pylori* 43504) or 18 hours (*S. Typhimurium* 700408, and *K. pneumoniae* BAA2472). Media only control contained only BHI-0.4% β -cyclodextrin (*H. pylori* 43504) or LB (*S. Typhimurium* 700408, and *K. pneumoniae* BAA2472). Determination of biofilm formation was measured by crystal violet staining. Error bars indicate standard deviation from 1 independent experiment with 3-8 replicates per condition.

FIG. 11 shows the antibiofilm effect of DMG against an established *H. pylori* biofilm. *H. pylori* 43504 cells were incubated in 96 well plates for 48 hours to allow for biofilm formation. DMG then was added to wells and incubated a further 24 hours. Determination of remaining biofilm was measured by crystal violet staining. Error bars indicate standard deviation from one experiment with 7-24 replicates per condition.

FIG. 12A – FIG. 12B shows the effect of DMG alone, or in combination with CuSO_4 , on the growth of *Campylobacter concisus* (FIG. 12A) or *Campylobacter jejuni* (FIG. 12B), as described in Example 4. *C. concisus* or *C. jejuni* cells were harvested, standardized to OD_{600} of 1, and serially

(10-fold) diluted in sterile 0.8% NaCl, before being spotted (5 µL) on solid media containing various concentrations of DMG or/and CuSO₄. Colony-forming units (CFUs) were counted after 24 hours incubation at 37°C under microaerobic conditions (for *C. jejuni*) or hydrogen-enriched microaerobic conditions (for *C. concisus*).

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DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

This disclosure describes compositions including dimethylglyoxime (DMG) and methods of using those compositions. In some aspects, this disclosure describes administering the composition including DMG to reduce the availability of nickel in the subject. In one aspect, this disclosure describes administering the composition to a subject suffering from or susceptible to a bacterial infection. In some embodiments, the bacterial infection may include a multi-drug resistant (for example, an antibiotic resistant) bacterium. In another aspect, this disclosure describes administering a composition including DMG to a subject suffering from or susceptible to a β-amyloid peptide aggregation. In a further aspect, this disclosure describes administering a composition including DMG to a subject suffering from or susceptible to a nickel allergy and/or an obese subject. In yet another aspect, this disclosure describes administering a composition including DMG to a subject to alter the balance of bacteria in the subject's microbiome. In some aspects, this disclosure describes using DMG or a composition including DMG to disrupt a biofilm or prevent biofilm formation.

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Metal chelation and bacteria

Nickel is required as a cofactor for several bacterial enzymes, including acireductone dioxygenase, [NiFe]-hydrogenase, glyoxalase I, superoxide dismutase, and urease (Benoit and Maier 2013 Nickel Ions in Biological Systems, p. 1501-1505 in Kretsinger *et al.* (eds.), Encyclopedia of Metalloproteins. Springer New York, New York, NY). The nickel requirement for enzymes associated only with bacterial (and not host) enzymes suggests nickel sequestration as a possible therapeutic target to combat several pathogens (see, for example, Rowinska-Zyrek *et al.* 2014. Dalton Trans 43:8976-8989); see also Table 1A – Table 1B. For instance, targeting nickel trafficking pathways to inactivate both the H₂-uptake [Ni-Fe] hydrogenase and the urease in the gastric pathogen *Helicobacter pylori* has been proposed (de Reuse *et al.* 2013 Front Cell Infect Microbiol 3:94; Maier 2003 Microbes Infect. 5:1159-1163).

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The nickel requirement for *Cryptococcus neoformans*'s urease has been identified as the fungus's "Achilles' heel" (Morrow *et al.* 2013 mBio 4(4):e00408-13). Furthermore, the host defense protein human calprotectin was recently shown to sequester nickel away from two pathogens, *Staphylococcus aureus* and *Klebsiella pneumoniae*, subsequently inhibiting their
 5 respective urease activity in bacterial culture (Nakashige *et al.* 2017 J Am Chem Soc 139:8828-8836). Many Enterobacteriaceae depend on nickel as a cofactor for their hydrogenase and/or urease enzymes. Hence, *Escherichia coli* and *Salmonella* species, including *S. enterica* serovar Typhimurium (referred to herein as *S. Typhimurium*), possess several Ni-containing hydrogenases (but not urease), while *Klebsiella* species, such as *K. pneumoniae*, possess a urease, as well as
 10 several hydrogenases. It has been shown that molecular hydrogen (H₂) use (by H₂-uptake [Ni-Fe] hydrogenases Hya, Hyb and Hyd) is essential for *S. Typhimurium* virulence (Maier *et al.* 2004 Infect Immun 72:6294-6299; Maier *et al.* 2014 PLoS One 9:e110187; Lamichhane-Khadka *et al.* 2015. Infect Immun 83:311-316.) Although it has not been formally demonstrated, H₂ metabolism has been hypothesized to be equally crucial for *K. pneumoniae*'s virulence. These results and
 15 predictions highlight the potential for Ni-chelation as an antibacterial therapy (Maier and Benoit, 2019 *Inorganics* 2019; 7:80) ; Benoit et al., 2020 MMBR 00092-19)

Use of metal chelators at the time of the invention

At the time of the invention, some metal chelators were already used (or were under
 20 evaluation in clinical trials) as drugs to control various human diseases, including cardiovascular diseases and Alzheimer's disease. Oral chelation is currently used to treat the hepatocellular copper inherited disorder known as Wilson disease. Furthermore, metal chelators can also be used to neutralize metal toxicity (Aaseth *et al.* 2015 J Trace Elem Med Biol 31:260-266; Sears 2013 Scientific World Journal 2013:219840), including nickel toxicity: for instance, the chelating agent
 25 sodium diethyldithiocarbamate (DCC) has been shown to be an effective drug against nickel carbonyl poisoning (Sunderman 1990 Ann Clin Lab Sci 20:12-21); likewise, disulfiram, a compound which is eventually metabolized in two DCC molecules, is FDA-approved to treat nickel carbonyl poisoning.

For some diseases there are clear benefits to chelation therapy, but sometimes the therapies
 30 have met with mixed results in benefiting the patient (Mathew *et al.* 2017 Cardiovasc Drugs Ther 31:619-625; Roberts *et al.* 2017 Handb Clin Neurol 142:141-156; Aaseth *et al.* 2015 J Trace Elem Med Biol 31:260-266); the latter is attributed in part to the toxic side effects of the chelating

chemicals (Aaseth *et al.* 2015 J Trace Elem Med Biol 31:260-266; Andersen *et al.* 2016 J Trace Elem Med Biol 38:74-80.) Hence, the use of “old” chelators such as ethylene diamine tetra acetate (EDTA) and 2,3-dimercaptopropanol (BAL) is now restricted, due to their toxicity (Aaseth *et al.* 2015 J Trace Elem Med Biol 31:260-266). The use of disulfiram is also controversial, since it has
5 been associated with elevated nickel levels in rat brains (Baselt *et al.* 1982. Res Commun Chem Pathol Pharmacol 38:113-124), as well as with hepatotoxicity in humans (Kaaber *et al.* 1979. Contact Dermatitis 5:221-228) and elevated nickel levels in body fluids of patients with chronic alcoholism (Hopfer *et al.* 1987 Res Commun Chem Pathol Pharmacol 55:101-109).

Table 1A

2017 WHO priority MDR pathogens with Ni-containing enzymes		Ni-containing enzyme				Comments
		Hydrogenase	Urease	Glyoxalase I	Aci. Dioxigenase	
		Uses or produces H ₂ (number in brackets →number of H ₂ ases)	Converts urea to CO ₂ and NH ₃	detoxifies methyl-glyoxal (metabolism byproduct)	converts 1,2-dihydroxy-5- (methylthio)pent-3-en-3-one to 3-(methylthio)propanoate formate and CO ₂	Hyp accessory proteins are needed for hydrogenase Ure accessory proteins are needed for urease GloA → glyoxalase (135 aa, very conserved) MtnD → acireductone dioxigenase
Priority 1: CRITICAL						
	Carbapenem ^R		✓	✓	✓	Ure; Only one HypB and one HycD homolog; GloA; MntD
	Carbapenem ^R	✓	✓	✓	✓	Hyp and Ure; GloA; MtnD
Carbapenem ^R 3rd gen. Cephalosporin ^R Enterobacteriaceae	<i>Klebsiella pneumoniae</i>		✓	✓	✓	Ure; GloA; MtnD
	<i>Escherichia coli</i>	✓ (4)		✓	✓	Hyp; GloA; MntD
	<i>Enterobacter</i> spp.	✓ (2)	✓	✓	✓	Hyp and Ure; GloA; MntD
	<i>Serratia</i> spp.		✓	✓	✓	Ure; GloA; MtnD
	<i>Proteus</i> spp.	✓	✓	✓	✓	Hyp and Ure; GloA; MtnD
	<i>Providencia</i> spp.	✓	✓	✓	✓	Hyp and Ure; GloA; MtnD
	<i>Morganella</i> spp.	✓	✓	✓		Hyp and Ure; GloA
Priority 2: HIGH						
	Vancomycin ^R		✓			Ure accessory and structural
	Methicillin ^R , Vancomycin ^R		✓			Prot annotated as "soluble hydrogenase", no match. Ure.
	Clarithromycin ^R	✓	✓			Hyp and Ure
	Fluroquinolone ^R	✓				Hyp
	Fluroquinolone ^R	✓ (4)		✓	✓	Hyp; GloA; MntD
	Cephalosporin ^R , Fluroquinolone ^R		✓?	✓		UreABC in <i>Neisseria</i> spp. (can't find hit in <i>N.gonorrhoeae</i>); GloA
Priority 3: MEDIUM						
	Penicillin ^R		✓	✓	✓	HyfB homolog; Ure accessory; GloA; MntD
	Ampicillin ^R		✓	✓		Ure accessory found; GloA
	Fluroquinolone ^R	✓ (3)	✓	✓		Hyp and Ure accessory found; GloA

Table 1B (Abbreviations: **Ard**: acireductone dioxxygenase; **Glo-I**: Glyoxalase I; **Hyc**: H₂-evolving hydrogenase; **Hyd**: H₂-uptake hydrogenase; **Sod**: superoxide dismutase; **Ure**: urease.)

	Pathogen	Ni-Enzyme	Pathogen	Ni-Enzyme
	EUKARYOTES			
	Human fungi		<i>Brucella melitensis</i>	Ure
5	<i>Cryptococcus neoformans</i>	Ure	<i>Brucella suis</i>	Ure
	<i>Cryptococcus gattii</i>	Ure	Betaproteobacteria	
	<i>Coccidioides posadasii</i>	Ure	<i>Neisseria meningitidis</i>	Glo-I
	<i>Histoplasma capsulatum</i>	Ure	<i>Neisseria gonorrhoeae</i>	Glo-I
	<i>Paracoccidioides brasiliensis</i>	Ure	Gamma proteobacteria	
10	Oomycetes		All γ-proteobacteria	Ard
	<i>Pythium insidiosum</i>	Ure	All γ-proteobacteria	Glo-I
	Protists		<i>Acinetobacter baumannii</i>	Ure
	<i>Leishmania major</i>	Glo-I	<i>Acinetobacter lwoffii</i>	Ure
	<i>Leishmania donovani</i>	Glo-I	<i>Actinobacillus</i>	Ure
15	<i>Trypanosoma cruzi</i>	Ard, Glo-I	<i>pleuropneumoniae</i>	Hyd-1
	PROKARYOTES		<i>Escherichia coli</i>	Hyd-2, Hyc
	Actinobacteria		<i>E. coli</i> (Shiga-toxin producing)	Ure
	<i>Actinomyces naeslundii</i>	Ure	<i>Edwardsiella tarda</i>	Hyd
20	<i>Corynebacterium urealyticum</i>	Ure	<i>Haemophilus influenzae</i>	Ure
	<i>Mycobacterium tuberculosis</i>	Hyc, Ure	<i>Klebsiella pneumoniae</i>	Ure
	<i>Streptomyces scabies</i>	Sod	<i>Morganella morganii</i>	Ure
	Firmicutes		<i>Proteus mirabilis</i>	Hyd, Ure
	<i>Clostridia</i>		<i>Providencia stuartii</i>	Ure
25	<i>Staphylococcus aureus</i>	Glo-I	<i>Pseudomonas aeruginosa</i>	Glo-I
	<i>Staphylococcus epidermidis</i>	Ure	<i>Salmonella Typhimurium</i>	Hyd-1, Hyd-2, Hyd-5, Hyc
	<i>Staphylococcus saprophyticus</i>	Ure	<i>Shigella flexneri</i>	Hyd
	<i>Streptococcus salivarius</i>	Ure	<i>Vibrio parahaemolyticus</i>	Ure
	Mollicutes		<i>Yersinia enterocolitica</i>	Ure
30	<i>Ureaplasma urealyticum</i>	Ure	<i>Yersinia pestis</i>	Glo-I
	<i>Ureaplasma parvum</i>	Ure	Deltaproteobacteria	
	<i>Ureaplasma diversum</i>	Ure	<i>Bilophila wadsworthia</i>	Hyd
	Proteobacteria		Epsilonproteobacteria	
	Alphaproteobacteria		<i>Campylobacter jejuni</i>	Hyd
35	<i>Brucella abortus</i>	Ure	<i>Campylobacter concisus</i>	Hyd
			<i>Helicobacter hepaticus</i>	Hyd, Ure
			<i>Helicobacter mustelae</i>	Ure
			<i>Helicobacter pylori</i>	Hyd, Ure

Dimethylglyoxime (DMG)

As further described in Example 1, nickel-specific chelation and the inhibition of bacterial growth was achieved *in vitro* and *in vivo* using dimethylglyoxime (DMG). Two molecules of DMG are needed to coordinate one Ni (II) molecule (FIG. 1A). DMG is reported to much prefer
5 complexation with nickel over other metals. The molecule was first described as nickel “precipitant” in 1946 (Minster 1946 Analyst 71:424-428) and was later used to identify nickel exposure of the skin (Choman 1962 Stain Technol 37:325-326), a procedure commonly known as “DMG test” (Thyssen *et al.* 2010. Contact Dermatitis 62:279-288; Julander *et al.* 2011 Contact Dermatitis 64:151-157).

10 DMG is also used to determine nickel levels in the environment (in soil, water, industrial effluents) (Ferancova *et al.* 2016 J Hazard Mater 306:50-57; Ershova *et al.* 2000 Fresenius J Anal Chem 367:210-211; Onikura *et al.* 2008 Environ Toxicol Chem 27:266-271) as well as to assess possible toxic levels of nickel in various items, including jewelry Thyssen *et al.* 2009. Sci Total Environ 407:5315-5318), mobile phones (Jensen *et al.* 2011. Contact Dermatitis 65:354-358) or
15 surgical items (Boyd *et al.* 2018 Dermatol Online J 24(4)).

DMG has also been used to remove nickel from laboratory supplies, growth media, or equipment (Benoit *et al.* 2013 Infect Immun 81:580-584) or from whole bacterial cells with the aim of studying roles of nickel in microbes. For example, studies on maturation of Ni-binding proteins (for example, hydrogenase and/or urease) in *H. pylori* (Maier 2003 Microbes Infect 5:1159-1163;
20 Seshadri *et al.* 2007 J Bacteriol 189:4120-4126; Saylor *et al.* 2018 Microbiology 164:1059-1068; Benanti *et al.* 2009 J Bacteriol 191:2405-2408) or in *Azotobacter chroococcum* (Partridge *et al.* 1982 Biochem J 204:339-344) have used DMG.

In 2007, the possible pathogen-inhibitory properties of an insoluble form of DMG (Sigma # D-1885 with formula weight of 116.12 g/mol) were tested. The DMG was administered as ethanolic
25 solutions, and the animals (BALB/C mice) appeared ill even when given chelator alone, exhibiting symptoms consistent with extreme constipation. Without wishing to be bound by theory, it is believed that upon stomach absorption of the ethanol, DMG came out of solution and caused intestinal compaction.

Methods of Using DMG

In one aspect, this disclosure describes a method that includes administering a chelator or a pharmaceutical composition including the chelator to a subject. In some embodiments, the chelator preferably includes dimethylglyoxime (DMG).

5 In some embodiments, the chelator or the pharmaceutical composition including the chelator may be administered to a subject to reduce the availability of metal in the subject. In some embodiments, the metal preferably includes nickel. In some embodiments, the metal includes copper.

10 In some embodiments, the chelator may be administered to a subject in an amount sufficient to reduce the availability of metal in the subject. In some embodiments, the chelator may be administered (including, for example, to a subject) in an amount sufficient to inhibit the growth of a pathogen. In some embodiments, the chelator may be administered to a subject in an amount sufficient to halt or slow the progression of a pathogenic infection or symptoms of a pathogenic infection within the subject.

15 In some embodiments, the DMG preferably includes soluble DMG. In some embodiments, soluble DMG preferably includes includes water soluble DMG. In some embodiments, the soluble DMG includes disodium salt DMG and/or disodium salt octahydrate DMG. In some embodiments, the soluble DMG preferably includes disodium salt octahydrate DMG. Given the previous results with insoluble DMG including the toxicity observed in mice, the high efficacy and low toxicity of
20 DMG described herein was particularly surprising.

A subject may include a human or an animal. An animal may include a companion animal, a domesticated animal such as a farm animal, an animal used for research, or an animal in the wild. Companion animals include, but are not limited to, dogs, cats, hamsters, gerbils, and guinea pigs. Domesticated animals include, but are not limited to, chickens, cattle, horses, pigs, goats, and
25 llamas. Research animals include, but are not limited to, mice, rats, dogs, apes, and monkeys.

In some embodiments, treatment of chickens with a chelator (for example, DMG) may be particularly desirable because infection of chickens with *C. jejuni* is a major cause of food poisoning. In some embodiments, DMG may be administered along with copper in chickens. As described in Example 4, while millimolar levels of DMG are bacteriostatic against *C. jejuni*, the
30 addition of micromolar levels of copper (II) surprisingly rendered millimolar levels of DMG bactericidal towards *C. jejuni*. Additionally, without wishing to be bound by theory, it is believed

that, because of the high levels of copper already present in the diets of many chickens, co-administration of additional copper may not be necessary to see the bactericidal effect of DMG.

Pharmaceutical Compositions

5 In another aspect, the present disclosure provides a composition including, for example, a pharmaceutical composition, that includes as an active agent, a chelator as described herein, and a pharmaceutically acceptable carrier. In some embodiments, the chelator includes DMG.

The active agent is formulated in a pharmaceutical composition and then, in accordance with the method of the invention, administered to an animal. In some embodiments, the animal is a
10 vertebrate, particularly a mammal, such as a human patient, companion animal, or domesticated animal, in a variety of forms adapted to the chosen route of administration. A pharmaceutical composition includes a composition suitable for oral, rectal, vaginal, topical, nasal, ophthalmic, or parenteral (including subcutaneous, intramuscular, intraperitoneal, and intravenous) administration.

The pharmaceutically acceptable carrier can include, for example, an excipient, a diluent, a
15 solvent, an accessory ingredient, a stabilizer, a protein carrier, or a biological compound. Non-limiting examples of a protein carrier includes keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA), ovalbumin, or the like. Non-limiting examples of a biological compound which can serve as a carrier include a glycosaminoglycan, a proteoglycan, and albumin. The carrier can be a synthetic compound, such as dimethyl sulfoxide or a synthetic polymer, such as a
20 polyalkyleneglycol. Ovalbumin, human serum albumin, other proteins, polyethylene glycol, or the like can be employed as the carrier. In some embodiments, the pharmaceutically acceptable carrier may include at least one compound that is not naturally occurring or a product of nature.

In some embodiments, the active agent may be formulated in combination with one or more additional active agents, such an antibacterial compound. Any known therapeutic agent can be
25 included as an additional active agent. The action of the additional active agent in the combination therapy can be cumulative to the chelator or it can be complementary, for example to manage side effects or other aspects of the patient's medical condition. In one embodiment, the combination therapy includes at least one compound that is not naturally occurring or a product of nature. In some embodiments, the combination therapy includes an antibiotic including, for example, an
30 antibiotic belonging to the β -lactam class (including, for example, penicillin derivatives, cephalosporins, or carbapenems), an antibiotic belonging to the macrolide class (including, for example, erythromycin, azithromycin, or clarithromycin), an antibiotic belonging to the

glycopeptide class (including, for example, vancomycin), an antibiotic belonging to the fluoroquinolone class (including, for example, ciprofloxacin or levofloxacin) or an antibiotic belonging to the aminoglycoside class (including, for example, gentamycin, neomycin, or streptomycin), or a combination thereof.

5 In some embodiments, the additional active agent preferably includes an antibacterial compound. In some embodiments, the antibacterial compound includes copper. In some embodiments, the additional active agent may include a metallic ion or an antibacterial compound that produces a metallic ion.

10 In some embodiments, when the antibacterial compound includes copper, the copper may include copper (I) or copper (II). In some embodiments, including, for example when a composition or compound is intended to be administered to a subject suffering from or susceptible to a *Campylobacter jejuni* infection, the metallic ion may preferably be Cu^{2+} . As described in Example 4, while millimolar levels of DMG are bacteriostatic against *C. jejuni*, surprisingly, the addition of micromolar levels of copper (II) renders millimolar levels of DMG bactericidal towards *C. jejuni*.
15 This effect was unexpected and was serendipitously discovered. While testing the ability of nickel to inhibit DMG-mediated growth inhibition of *C. jejuni*, zinc and copper were used as a control – but, rather than copper having no effect of the ability of DMG to inhibit the growth of *C. jejuni*, an increased effect on the ability of DMG to inhibit the growth of *C. jejuni* was observed instead.

20 In some embodiments, the metallic ion is preferably a divalent cation. Exemplary divalent cations include Cu^{2+} (Cu(II)); cobalt (II) (Co^{2+}); manganese (II) (Mn^{2+}); zinc (II) (Zn^{2+}); and tin (Sn^{2+}). In some embodiments, the divalent cation does not include nickel, or, because of their toxicity to subjects, Cadmium (Cd^{2+}), mercury (Hg^{2+}) or lead (Pb^{2+}).

25 In embodiments when the antibacterial compound produces a metallic ion, the compound may preferably be a salt or another compound that produces a metal ion upon being dissolved, for example, in water or another pharmaceutical carrier. Exemplary salts include, CuSO_4 , CoCl_2 , MnSO_4 , and ZnSO_4 .

30 The formulations can be conveniently presented in unit dosage form and can be prepared by any of the methods well-known in the art of pharmacy. In some embodiments, a method includes the step of bringing the active agent into association with a pharmaceutical carrier. In general, the formulations are prepared by uniformly and intimately bringing the active compound into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product into the desired formulations.

Formulations of the present disclosure suitable for oral administration can be presented as discrete units such as tablets, troches, capsules, lozenges, wafers, or cachets, each containing a predetermined amount of the active agent as a powder or granules, as liposomes, or as a solution or suspension in an aqueous liquor or non-aqueous liquid such as a syrup, an elixir, an emulsion, or a draught. The tablets, troches, pills, capsules, and the like can also contain one or more of the following: a binder such as gum tragacanth, acacia, corn starch, or gelatin; an excipient such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid, and the like; a lubricant such as magnesium stearate; a sweetening agent such as sucrose, fructose, lactose, or aspartame; and a natural or artificial flavoring agent. When the unit dosage form is a capsule, it can further contain a liquid carrier, such as a vegetable oil or a polyethylene glycol. Various other materials can be present as coatings or to otherwise modify the physical form of the solid unit dosage form. For instance, tablets, pills, or capsules can be coated with gelatin, wax, shellac, sugar, and the like. A syrup or elixir can contain one or more of a sweetening agent, a preservative such as methyl- or propylparaben, an agent to retard crystallization of the sugar, an agent to increase the solubility of any other ingredient, such as a polyhydric alcohol, for example glycerol or sorbitol, a dye, and flavoring agent. The material used in preparing any unit dosage form is substantially nontoxic in the amounts employed. The active agent can be incorporated into preparations and devices in formulations that may or may not be designed for sustained release.

Formulations suitable for parenteral administration conveniently include a sterile aqueous preparation of the active agent, or dispersions of sterile powders of the active agent, which are preferably isotonic with the blood of the recipient. Parenteral administration of a chelator (for example, through an intravenous drip) is one form of administration. Isotonic agents that can be included in the liquid preparation include sugars, buffers, and sodium chloride. Solutions of the active agent can be prepared in water, optionally mixed with a nontoxic surfactant. Dispersions of the active agent can be prepared in water, ethanol, a polyol (such as glycerol, propylene glycol, liquid polyethylene glycols, and the like), vegetable oils, glycerol esters, and mixtures thereof. The ultimate dosage form is sterile, fluid, and stable under the conditions of manufacture and storage. The necessary fluidity can be achieved, for example, by using liposomes, by employing the appropriate particle size in the case of dispersions, or by using surfactants. Sterilization of a liquid preparation can be achieved by any convenient method that preserves the bioactivity of the active agent, preferably by filter sterilization. Preferred methods for preparing powders include vacuum drying and freeze drying of the sterile injectable solutions. Subsequent microbial contamination can

be prevented using various antimicrobial agents, for example, antibacterial, antiviral and antifungal agents including parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. Absorption of the active agents over a prolonged period can be achieved by including agents for delaying, for example, aluminum monostearate and gelatin.

5 Nasal spray formulations include purified aqueous solutions of the active agent with preservative agents and isotonic agents. Such formulations are preferably adjusted to a pH and isotonic state compatible with the nasal mucous membranes. Formulations for rectal or vaginal administration can be presented as a suppository with a suitable carrier such as cocoa butter, or hydrogenated fats or hydrogenated fatty carboxylic acids. Ophthalmic formulations are prepared by
10 a similar method to the nasal spray, except that the pH and isotonic factors are preferably adjusted to match that of the eye. Topical formulations include the active agent dissolved or suspended in one or more media such as mineral oil, petroleum, polyhydroxy alcohols, or other bases used for topical pharmaceutical formulations. Topical formulations can be provided in the form of a bandage, wherein the formulation is incorporated into a gauze or other structure and brought into
15 contact with the skin.

Administration

A chelator (including, for example, DMG) as the active agent, can be administered to a subject alone or in a pharmaceutical composition that includes the active agent and a
20 pharmaceutically acceptable carrier. The active agent is administered to an animal in an amount effective to produce the desired effect. A chelator can be administered in a variety of routes, including orally, parenterally, intraperitoneally, intravenously, intraarterially, transdermally, sublingually, intramuscularly, rectally, transbuccally, intranasally, liposomally, via inhalation, vaginally, intraocularly, via local delivery by catheter or stent, subcutaneously, intraadiposally,
25 intraarticularly, intrathecally, or in a slow release dosage form.

A formulation or pharmaceutical composition including the chelator can be administered as a single dose or in multiple doses. Useful dosages of the active agent can be determined by comparing their *in vitro* activity and the *in vivo* activity in animal models. Methods for extrapolation of effective dosages in mice, and other animals, to humans are known in the art.

30 Dosage levels of the active agent in the pharmaceutical compositions of this disclosure can be varied to obtain an amount of the active agent which is effective to achieve the desired therapeutic response for a particular subject, composition, and mode of administration, without

being toxic to the subject. The selected dosage level will depend upon a variety of factors including the activity of the particular compound of the present disclosure employed, or the ester, salt or amide thereof, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the chelator, the age, sex, weight, condition, general health, and prior medical history of the subject being treated, and like factors well known in the medical arts.

Dosages and dosing regimens that are suitable for other chelators may be suitable for therapeutic or prophylactic administration of the chelators of the present disclosure (including, for example, DMG). Dosages or dosing regimens in use for other chelators, including, for example, sodium diethyldithiocarbamate (DCC), disulfiram, ethylene diamine tetra acetate (EDTA), and 2,3-dimercaptopropanol (BAL), may serve as guideposts for developing suitable animal and human dosages and dosing regimens.

In an exemplary embodiment, the chelator (for example, DMG) may be administered to a subject in an amount of at least 5 mg/kg, at least 10 mg/kg, at least 20 mg/kg, at least 30 mg/kg, at least 40 mg/kg, at least 50 mg/kg, at least 60 mg/kg, at least 70 mg/kg, at least 80 mg/kg, at least 90 mg/kg, or at least 100 mg/kg. In exemplary embodiment, the chelator may be administered to a subject in an amount of up to 40 mg/kg, up to 50 mg/kg, up to 60 mg/kg, up to 70 mg/kg, up to 80 mg/kg, up to 90 mg/kg, up to 100 mg/kg, up to 500 mg/kg, or up to 1000 mg/kg. In an exemplary embodiment, DMG may be administered to a subject orally, intravenously, or intramuscularly.

A physician or veterinarian having ordinary skill in the art can readily determine and prescribe the effective amount of the pharmaceutical composition required. For example, the physician could start doses of the chelator employed in the pharmaceutical composition at levels lower than that required in order to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect is achieved.

Bacterial Infections

In some embodiments, the chelator (including, for example, DMG) or pharmaceutical composition including the chelator may be administered to a subject suffering from or susceptible to a bacterial infection. Additionally or alternatively, a chelator (including, for example, DMG) may be used to treat or prevent a pathogenic infection.

A pathogenic infection may include, for example, a bacterial infection, a fungal infection, or an infection caused by a non-fungal eukaryotic pathogen. Exemplary pathogens that may be nickel-

dependent are provided in Maier and Benoit *Inorganics* 2019; 7:80. In some embodiments, a pathogen that includes a nickel-containing enzyme includes a pathogen listed in Table 1A. In some embodiments, a pathogen that includes a nickel-containing enzyme includes a pathogen listed in Table 1B.

5 Exemplary bacterial infections include but are not limited to an infection with a bacterium that includes a nickel-containing enzyme. In some embodiments, a bacterium that includes a nickel-containing enzyme includes a multi-drug resistant pathogen. In some embodiments, a bacterium that includes a nickel-containing enzyme may include *Acinetobacter baumannii*, *Enterococcus faecium*, *Escherichia coli*, *Helicobacter pylori*, *Haemophilus influenzae*, *Neisseria gonorrhoeae*,
 10 *Streptococcus pneumoniae*, a *Campylobacter* species, an *Enterobacter* species, a *Klebsiella* species (including, for example, *K. pneumoniae*), a *Morganella* species, a *Proteus* species, a *Providencia* species, a *Pseudomonas* species (including, for example, *Pseudomonas aeruginosa*), a *Salmonella* species (including, for example, *S. enterica* serovar Typhi and *S. enterica* serovar Typhimurium), a *Serratia* species, a *Shigella* species, or a *Staphylococcus* species (including, for example,
 15 *Staphylococcus aureus*).

Exemplary fungal infections include but are not limited to fungi that include a nickel-containing enzyme. In some embodiments, a fungus that includes a nickel-containing enzyme may include *Cryptococcus neoformans*, *Cryptococcus gattii*, *Coccidioides posadasii*, *Histoplasma capsulatum*, or *Paracoccidioides brasiliensis*.

20 Exemplary infections caused by a non-fungal eukaryotic pathogen include but are not limited to eukaryotes that include a nickel-containing enzyme. In some embodiments, a eukaryote that include a nickel-containing enzyme may include *Pythium insidiosum*, *Leishmania major*, *Leishmania donovani*, or *Trypanosoma cruzi*.

Exemplary nickel-containing enzymes include a hydrogenase, a urease, a Glyoxalase I, a
 25 acireductone dioxygenase, a superoxide dismutase etc. In some embodiments, the bacteria may further include an accessory protein. An accessory protein involved in Ni-dependent hydrogenase maturation may include, for example, a Hyp protein (for example, HypA, HypB, HypC, HypD, HypE, HypF, or HypG) or a homolog thereof. An accessory protein involved urease activation may include, for example, a Ure protein (for example, UreD, UreF, UreG, or UreH).

30 In some embodiments, a chelator can also be administered prophylactically, to prevent or delay the development of infection with a bacterium. Treatment that is prophylactic, for instance, can be initiated before a subject manifests symptoms of infection with a bacterium. An example of a

subject that is at particular risk of developing a bacterial infection is an immunocompromised person. Treatment can be performed before, during, or after the diagnosis or development of symptoms of infection. Treatment initiated after the development of symptoms may result in decreasing the severity of the symptoms of one of the conditions, or completely removing the symptoms. A chelator can be introduced into the subject at any stage of bacterial infection.

Administration of a chelator can occur before, during, and/or after other treatments. Such combination therapy can involve the administration of a chelator during and/or after the use of other anti-bacterial agents. The administration a chelator can be separated in time from the administration of other anti-bacterial agents by hours, days, or even weeks.

In some embodiments, the chelator is administered to a subject that has been diagnosed with, or is exhibiting symptoms of, or is at risk of developing, a bacterial infection. In another embodiment, the chelator is administered in a subject or subject population that serves, may serve, or is suspected of serving as an infection reservoir, regardless of the presence of symptoms. Administration can be, for example, part of a small or large scale public health infection control program. The chelator can, for example, be added to animal feed as a prophylactic measure for reducing, controlling or eliminating fungal infection in a wild or domestic animal population. The compound can, for example, be administered as part of routine or specialized veterinary treatment of a companion or domesticated animal or animal population. It should be understood that administration of the chelator can be effective to reduce or eliminate bacterial infection or the symptoms associated therewith; to halt or slow the progression of infection or symptoms within a subject; and/or to control, limit, or prevent the spread of infection within a population, or movement of infection to another population.

Metal-Related β -Amyloid Peptide Aggregation

In some embodiments, the chelator or pharmaceutical composition including the chelator may be administered to a subject suffering from or susceptible to β -amyloid peptide aggregation. For example, in some embodiments, the subject may be suffering from or susceptible to Alzheimer's Disease (AD), adult Down Syndrome, or some types of cancers.

As shown in Example 2, DMG can inhibit the metal-mediated aggregation (especially nickel-mediated aggregation) of β -amyloid peptide. Indeed, DMG inhibits the aggregation of β -amyloid peptide even without the additional of metals (see FIG. 7) suggesting that metal bound to β -amyloid peptide may be causing "background" levels of aggregation.

In some embodiments, a chelator can also be administered prophylactically, to prevent or delay the development of β -amyloid peptide aggregation. Treatment that is prophylactic, for instance, can be initiated before a subject manifests symptoms of β -amyloid peptide aggregation and/or dementia. An example of a subject that is at particular risk of developing a β -amyloid peptide aggregation is a person with a family history of Alzheimer's Disease or the presence of a genetic mutation associated with Alzheimer's Disease. Treatment can be performed before, during, or after the diagnosis or development of symptoms of dementia. Treatment initiated after the development of symptoms may result in decreasing the severity of the symptoms of one of the conditions, or completely removing the symptoms. A chelator can be introduced into the subject at any stage of dementia.

Administration of a chelator can occur before, during, and/or after other treatments. Such combination therapy can involve the administration of a chelator during and/or after the use of other anti-dementia drugs, anti-Alzheimer's drugs, and/or drugs that prevent β -amyloid peptide aggregation. The administration a chelator can be separated in time from the administration of other agents by hours, days, or even weeks.

In some embodiments, the chelator is administered to a subject that has been diagnosed with, or is exhibiting symptoms of, or is at risk of developing β -amyloid peptide aggregation. It should be understood that administration of the chelator can be effective to reduce or eliminate β -amyloid peptide aggregation or the symptoms associated therewith and/or to halt or slow the progression of β -amyloid peptide aggregation or symptoms within a subject.

Alteration of a Subject's Microbiome and/or Obesity

In some embodiments, the chelator or pharmaceutical composition including the chelator may be administered to a subject suffering from or susceptible to a nickel allergy. In some embodiments, the subject may be obese. In some embodiments, the chelator or pharmaceutical composition including the chelator may be administered to a subject to alter the balance of bacteria in the subject's microbiome.

Recent studies have isolated nickel-resistance bacteria from the human microbiome (Lusi *et al.* 2017 New Microbe and New Infect 19: 67–70) and have shown that obese individuals with a nickel allergy have altered metabolisms compared to non-allergic, weight-matched individuals (Watanabe *et al.* 2018 PLoS ONE 13(8): e0202683). These finding suggest that chelation of nickel in nickel-allergic individuals and/or individuals with nickel-resistance bacteria in their microbiome

may provide a therapeutic benefit for those individuals including, for example, by altering the balance of bacteria in their microbiome and/or the individual's metabolism. The human intestinal microbiota (the intestinal microbiome) impacts many areas of human health, while some of the commensal bacteria in the gut use nickel as a component of enzymes. Therefore, alteration of nickel levels via nickel chelation is expected to alter intestinal microbiome content, potentially providing health benefits.

Administration of a chelator can occur before, during, and/or after other treatments. Such combination therapy can involve the administration of a chelator during and/or after the use of other treatments for nickel allergy. In an exemplary embodiment, DMG may be administered with another chelator including, for example, EDTA. The administration of a chelator can be separated in time from the administration of another agent by hours, days, or even weeks.

Inhibition or Disruption of Biofilms

In some embodiments, the chelator or a composition including the chelator may be used to prevent biofilm formation or to disrupt a biofilm. Treatment of a surface initiated after the development of a biofilm may result in the death of some of the bacterial cells within the biofilm or all of the bacterial cells within the biofilm. Treatment of a surface initiated before the development of a biofilm may result in the prevention of the biofilm or slowing the growth of a biofilm.

In some embodiments, the biofilm may include a *Campylobacter* species (including, for example, *C. jejuni* and/or *C. concisus*), *Helicobacter pylori*, a *Klebsiella* species, (including, for example, *Klebsiella pneumoniae*), a *Proteus* species, a *Pseudomonas* species (including, for example, *Pseudomonas aeruginosa*), a *Salmonella* species (including, for example, *S. Typhimurium*), or a *Staphylococcus* species (including, for example, *Staphylococcus aureus*), or a combination thereof. In some embodiments, the biofilm may include catheter-associated bacteria and/or medical tubing-associated bacteria.

In some embodiments, the biofilm may be present on a medical device including, for example, a catheter, medical tubing, and/or an indwelling device. Additional specific examples of medical devices on which a biofilm may be present include but are not limited to a nasogastric tube, a urinary catheter, a central venous catheter, an umbilical line, an endotracheal tube, a contact lens, a heart valve, a prosthetic device, etc. In some embodiments, a medical device and/or a surface of a medical device may be pre-treated to prevent the formation of a biofilm or to slow the growth of a biofilm.

In some embodiments, treatment of a surface includes application and/or deposition of the chelator to the surface; ionic binding of the chelator to the surface; or incorporation of the chelator to a polymeric matrix bound to the surface.

Bacterial biofilms often prevent or reduce clearance by antibiotics and enable persistent infections in patients. Yonezawa *et al.* 2013 PLoS One 8:e73301; Davies D. 2003 Nature Reviews Drug discovery 2:114; Vuotto *et al.* 2017 Journal of Applied Microbiology 123:1003-1018; Stewart 2015 Microbiology Spectrum 3(3). Data described in Example 3 indicates that DMG has biofilm-inhibitory properties against multidrug resistant *Salmonella* and *Klebsiella* strains, as well as against the gastric pathogen *Helicobacter pylori*.

In some embodiments, a composition including the chelator may include an additional active agent, such an antibacterial compound. In some embodiments the surface being treated with the chelator or a composition including the chelator may preferably include an additional active agent preferably includes an antibacterial compound.

In some embodiments, the additional active agent preferably includes an antibacterial compound. In some embodiments, the antibacterial compound includes copper. In some embodiments, the additional active agent may include a metallic ion or a antibacterial compound that produces a metallic ion.

In some embodiments, when the antibacterial compound includes copper, the copper may include copper (I) or copper (II). In some embodiments, including, for example when the chelator or a composition including the chelator is being used to prevent biofilm formation including *Campylobacter jejuni* or to disrupt a biofilm including *C. jejuni*, the metallic ion may preferably be Cu^{2+} . As described in Example 4, while millimolar levels of DMG are bacteriostatic against *C. jejuni*, the addition of micromolar levels of copper (II) renders millimolar levels of DMG bactericidal towards *C. jejuni*.

Exemplary Administration Embodiments

1. A method comprising administering a compound to a subject to reduce the availability of nickel in the subject, wherein the compound comprises dimethylglyoxime (DMG).
- 5 2. The method of Embodiment 1, wherein the subject comprises a human or an animal.
3. The method of Embodiment 2, wherein the animal comprises a chicken.
4. The method of any one of the preceding Embodiments, wherein the subject is suffering from or
10 susceptible to a pathogenic infection.
5. The method of Embodiment 4, wherein the pathogenic infection comprises infection with a bacterium that comprises a nickel-containing enzyme.
- 15 6. The method of Embodiment 4 or Embodiment 5, wherein the pathogenic infection comprises infection with *Acinetobacter baumannii*, *Enterococcus faecium*, *Escherichia coli*, *Helicobacter pylori*, *Haemophilus influenzae*, *Neisseria gonorrhoeae*, *Streptococcus pneumoniae*, a *Campylobacter* species, an *Enterobacter* species, a *Klebsiella* species, a *Morganella* species, a *Proteus* species, a *Providencia* species, a *Pseudomonas* species, a *Salmonella* species, a *Serratia*
20 species, a *Shigella* species, or a *Staphylococcus* species, or a combination thereof.
7. The method of Embodiment 4, wherein the pathogenic infection comprises infection with a fungus that comprises a nickel-containing enzyme.
- 25 8. The method of Embodiment 4 or Embodiment 7, wherein the pathogenic infection comprises infection with *Cryptococcus neoformans*, *Cryptococcus gattii*, *Coccidioides posadasii*, *Histoplasma capsulatum*, or *Paracoccidioides brasiliensis*, or a combination thereof.
9. The method of Embodiment 4, wherein the pathogenic infection comprises infection with a non-
30 fungal eukaryotic pathogen that comprises a nickel-containing enzyme.

10. The method of Embodiment 4 or Embodiment 9, wherein the pathogenic infection comprises infection with *Pythium insidiosum*, *Leishmania major*, *Leishmania donovani*, or *Trypanosoma cruzi*, or a combination thereof.

5 11. The method of any one of Embodiments 4 to 11, wherein the pathogenic infection comprises infection with a multi-drug resistant pathogen.

12. The method of Embodiment 1 or 2, wherein the subject is suffering from or susceptible to β -amyloid peptide aggregation.

10

13. The method of Embodiment 12, wherein the subject is suffering from or susceptible to Alzheimer's Disease or Down Syndrome or both.

14. The method of Embodiment 1 or 2, wherein the subject is suffering from or susceptible to a
15 nickel allergy.

15. The method of Embodiment 14, wherein the subject comprises nickel-resistance bacteria in their microbiome.

20 16. The method of any one of the preceding Embodiments, wherein the DMG comprises soluble DMG.

17. The method of Embodiment 16, wherein the soluble DMG comprises disodium salt DMG and/or disodium salt octahydrate DMG.

25

18. The method of any one of the preceding Embodiments, wherein the method further comprises administering an additional active agent to the subject.

19. The method of Embodiment 18, wherein the additional active agent comprises a metallic ion or
30 an antibacterial compound that produces a metallic ion.

20. The method of Embodiment 19, wherein the metallic ion comprises a divalent cation.

21. The method of any one of Embodiments 18 to 20, wherein the additional active agent comprises copper.

5 **Exemplary Methods of Treating Pathogenic Infection Embodiments**

1. A method of treating or preventing a pathogenic infection in a subject, the method comprising administering dimethylglyoxime (DMG) to the subject.

2. The method of Embodiment 1, wherein the DMG comprises soluble DMG.

10

3. The method of Embodiment 2, wherein the soluble DMG comprises disodium salt DMG and/or disodium salt octahydrate DMG.

15

4. The method of any one of the preceding Embodiments, wherein the subject comprises a human or an animal.

5. The method of Embodiment 4, wherein the animal comprises a chicken.

20

6. The method of any one of the preceding Embodiments, wherein the pathogenic infection comprises infection with a bacterium that includes a nickel-containing enzyme.

25

7. The method of any one of Embodiments, wherein the bacterial infection comprises infection with *Acinetobacter baumannii*, *Enterococcus faecium*, *Escherichia coli*, *Helicobacter pylori*, *Haemophilus influenzae*, *Neisseria gonorrhoeae*, *Streptococcus pneumoniae*, a *Campylobacter* species, an *Enterobacter* species, a *Klebsiella* species, a *Morganella* species, a *Proteus* species, a *Providencia* species, a *Pseudomonas* species, a *Salmonella* species, a *Serratia* species, a *Shigella* species, or a *Staphylococcus* species, or a combination thereof.

30

8. The method of any one of the preceding Embodiments, wherein the pathogenic infection comprises infection with a fungus that comprises a nickel-containing enzyme.

9. The method of one of the preceding Embodiments, wherein the pathogenic infection comprises infection with *Cryptococcus neoformans*, *Cryptococcus gattii*, *Coccidioides posadasii*, *Histoplasma capsulatum*, or *Paracoccidioides brasiliensis*, or a combination thereof.

5 10. The method of one of the preceding Embodiments, wherein the pathogenic infection comprises infection with a non-fungal eukaryotic pathogen that comprises a nickel-containing enzyme.

11. The method of one of the preceding Embodiments, wherein the pathogenic infection comprises infection with *Pythium insidiosum*, *Leishmania major*, *Leishmania donovani*, or *Trypanosoma*
10 *cruzi*, or a combination thereof.

12. The method of any one of the preceding Embodiments, wherein the pathogenic infection comprises infection with a multi-drug resistant pathogen.

15 13. The method of any one of the preceding Embodiments, wherein DMG is administered in combination with another anti-bacterial therapy.

14. The method of any one of the preceding Embodiments, wherein DMG is administered orally or intravenously.

20

15. The method of Embodiment 8, wherein the method comprises administering DMG orally, and wherein the DMG is presented as a capsule.

16. The method of any one of the preceding Embodiments, wherein the method further comprises
25 administering an additional active agent to the subject.

17. The method of Embodiment 16, wherein the additional active agent comprises a metallic ion or an antibacterial compound that produces a metallic ion.

30 18. The method of Embodiment 17, wherein the metallic ion comprises a divalent cation.

19. The method of any one of Embodiments 16 to 18, wherein the additional active agent comprises copper.

Exemplary Methods of Treating β -Amyloid Peptide Aggregation

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1. A method of treating or preventing β -Amyloid peptide aggregation in a subject, the method comprising administering dimethylglyoxime (DMG) to the subject.

2. The method of Embodiment 1, wherein the DMG comprises soluble DMG.

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3. The method of Embodiment 2, wherein the soluble DMG comprises disodium salt DMG and/or disodium salt octahydrate DMG.

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4. The method of any one of the preceding Embodiments, wherein the subject is suffering from or susceptible to Alzheimer's Disease or Down Syndrome or both.

5. The method of any one of the preceding Embodiments, wherein DMG is administered in combination with another anti-dementia therapy.

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6. The method of any one of the preceding Embodiments, wherein DMG is administered orally or intravenously.

7. The method of Embodiment 6, wherein the method comprises administering DMG orally, and wherein the DMG is presented as a capsule.

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Exemplary Methods of Treating Obesity and/or Nickel Allergy

1. A method of treating or preventing nickel allergy in a subject, the method comprising administering dimethylglyoxime (DMG) to the subject.

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2. A method of treating or preventing obesity in a subject, the method comprising administering dimethylglyoxime (DMG) to the subject.

3. A method of altering the balance of bacteria in the subject's microbiome, the method comprising administering dimethylglyoxime (DMG) to the subject.

5 4. The method of any one of the preceding Embodiments, wherein the DMG comprises soluble DMG.

5. The method of Embodiment 4, wherein the soluble DMG comprises disodium salt DMG and/or disodium salt octahydrate DMG.

10 6. The method of any one of the preceding Embodiments, wherein DMG is administered in combination with another treatment for a nickel allergy.

15 7. The method of any one of the preceding Embodiments, wherein DMG is administered orally or intravenously.

Exemplary Methods of Disrupting a Biofilm or Preventing Biofilms

20 1. A method of disrupting a biofilm or preventing biofilm formation, the method comprising treating a surface with dimethylglyoxime (DMG).

2. The method of Embodiment 1, wherein the biofilm comprises a *Campylobacter* species, *Helicobacter pylori*, a *Klebsiella* species, a *Proteus* species, a *Pseudomonas* species, a *Salmonella* species, or a *Staphylococcus* species, or a combination thereof.

25 3. The method of Embodiment 1 or 2, wherein the method comprises treating a surface after the formation of a biofilm, wherein treatment results in the death of bacterial cells within the biofilm.

30 4. The method of any one of the preceding Embodiments, wherein the method further comprises treating the surface with an additional active agent.

5. The method of any one of the preceding Embodiments, wherein the surface further comprises an additional active agent.

6. The method of Embodiment 5 or 6, wherein the additional active agent comprises a metallic ion or an antibacterial compound that produces a metallic ion.

7. The method of Embodiment 6 wherein the metallic ion comprises a divalent cation.

8. The method of any one of Embodiments 4 to 7, wherein the additional active agent comprises copper.

Exemplary Pharmaceutical Compositions

1. A pharmaceutical composition comprising a chelator, the chelator comprising soluble DMG.

2. The pharmaceutical composition of Embodiment 1, wherein the soluble DMG comprises disodium salt DMG and/or disodium salt octahydrate DMG.

3. The pharmaceutical composition of any one of the preceding embodiments, the chelator further comprising EDTA.

4. The pharmaceutical composition of any one of the preceding embodiments, wherein the pharmaceutical composition is formulated for oral or intravenous administration.

5. The pharmaceutical composition of any one of the preceding embodiments, wherein the pharmaceutical composition further comprises an additional active agent.

6. The pharmaceutical composition of Embodiment 5, wherein the additional active agent comprises a metallic ion or an antibacterial compound that produces a metallic ion.

7. The pharmaceutical composition of Embodiment 6, wherein the metallic ion comprises a divalent cation.

8. The pharmaceutical composition of any one of Embodiments 5 to 7, wherein the additional active agent comprises copper.

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The present invention is illustrated by the following examples. It is to be understood that the particular examples, materials, amounts, and procedures are to be interpreted broadly in accordance with the scope and spirit of the invention as set forth herein.

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EXAMPLES

Example 1 – Nickel chelation therapy as an approach to combat multi-drug resistant enteric pathogens

15 **RESULTS AND DISCUSSION**

The nickel-specific chelator DMG inhibits growth of various Enterobacteriaceae.

The inhibitory effect of DMG on bacterial growth was tested on three strains: MDR *K. pneumoniae* (ATCC BAA2472), MDR *S. Typhimurium* (ATCC 700408), and *S. Typhimurium* (ATCC 14028), which is a mouse colonizing strain (Gunn *et al.* 2000 Infect Immun 68:6139-6146).
20 Cells were inoculated at a starting OD₆₀₀ of 0.005 (approximately 5×10^6 CFU/mL) and grown for 24 hours at 37°C under aerobic conditions with vigorous shaking in presence of defined concentrations of DMG, as indicated, and the growth yield (CFU/mL) was determined after serial dilutions and plating (FIG. 2). While there was no measurable effect of DMG at 2.5 mM, 5 mM, or 7.5 mM on bacterial growth, more than 99.99% growth inhibition was achieved in presence of
25 10 mM chelator for all three bacterial strains tested. Since approximately 10^6 cells per mL were still detected, these results suggest DMG has a bacteriostatic effect on the growth of these Enterobacteriaceae. Thus, millimolar concentrations of DMG can inhibit *in vitro* growth of various Enterobacteriaceae, including MDR strains of *K. pneumoniae* and *S. Typhimurium*.

30 **Sublethal DMG levels abolish *S. Typhimurium* hydrogenase activity.**

To study the effect of Ni-chelation on hydrogenase activity in *S. Typhimurium*, cells from strain 14028 were grown for 6 hours on blood-based media containing increasing concentrations of DMG,

under H₂-enriched microaerobic atmosphere; these conditions (blood medium and H₂) have been previously shown to be favorable for the expression of all three *S. Typhimurium* respiratory hydrogenases (Maier *et al.* 2004 Infect Immun 72:6294-6299). Hydrogenase assays were carried out on whole cells using an amperometric method, as previously described (Maier *et al.* 2004 Infect Immun 72:6294-6299; Lamichhane-Khadka *et al.* 2015 Infect Immun 83:311-316). While addition of 0.1 mM DMG to the medium had no effect on the (combined) H₂-uptake hydrogenase activity, supplementation of the growth medium with either 0.5 mM, 1 mM, or 5 mM DMG significantly decreased hydrogenase activity, and addition of 10 mM DMG abolished hydrogenase activity (Table 2). The addition of 50 μ M NiCl₂ to a medium containing 1 mM DMG restored some hydrogenase activity (50% increase compared to the 1 mM DMG medium). Taken together, these results indicate that DMG inhibits hydrogenase activity in *S. Typhimurium*, probably through Ni-chelation. H₂-uptake hydrogenase activity is required for colonization in a murine model. Hence, the inhibitory effect of DMG on hydrogenase activity observed herein suggests the Ni-chelator could inhibit *S. Typhimurium* growth in animals.

Sublethal DMG levels inhibit MDR *Klebsiella pneumoniae* urease.

To study the effect of Ni-chelation on urease activity in MDR *K. pneumoniae* BAA-2472, cells were grown overnight in LB broth supplemented with sublethal concentrations of DMG, cells were harvested, broken and urease assays were performed on cell-free extracts (Table 3).

Supplementation of the growth medium with 1 mM or 2 mM DMG significantly decreased urease activity in MDR *K. pneumoniae*, while addition of 5 mM completely inhibited the urease activity in the pathogenic strain. Thus, similar to hydrogenase inhibition, it appears DMG-mediated Ni chelation can be used to efficiently block urease activity in *Klebsiella*. This confirms previous results from Nakashige and coworkers, who showed that calprotectin-driven chelation of nickel led to urease inhibition in *K. pneumoniae* (Nakashige *et al.* 2017 J Am Chem Soc 139:8828-8836). Urease is used in nitrogen metabolism by *K. pneumoniae*; when tested in a competition experiment with the wild-type strain, an isogenic urease mutant failed to colonize mouse intestines (Maroncle *et al.* 2006 Res Microbiol 157:184-193). Thus, the inhibition of *K. pneumoniae* urease by DMG, as shown in the present study, is anticipated to have a major (inhibitory) impact on the *in vivo* colonization of the pathogen.

Table 2. Effect of DMG chelation on hydrogenase activity in *S. Typhimurium* 14028.

DMG (mM) ^a	NiCl ₂ (mM)	Hydrogenase activity ^b
0		14.7 ± 2.8
0.1		14.0 ± 2.7
0.5		4.6 ± 1.2
1		3.9 ± 0.9
1	0.05	6.9 ± 1.7
5		0.8 ± 0.1
10		ND

^a DMG was added to a blood-based medium, and cells were grown for 6 hours under H₂-enriched microaerobic conditions before being harvested.

^b nmoles H₂ oxidized per minute per 10⁹ cells

Values shown are the mean ± standard deviation for 6 independent replicates.

Results beginning with 0.5 mM of DMG are significantly less than without DMG ($P < 0.01\%$, Student's *t*-test).

Table 3. Effect of DMG chelation on urease activity in *K. pneumoniae* BAA-2472

DMG (mM) ^a	Urease activity ^b
0	0.17 ± 0.03
1	0.05 ± 0.01
2	0.03 ± 0.01
5	ND ^c

^a DMG was added to LB broth, cells were grown overnight, and urease assays were performed on cell-free extracts using the phenol-hypochlorite method of Weatherburn *et al.* 1967 Analytical Chemistry 39:971-974.

^b Urease activity is expressed in μmoles of NH₃ produced per minute per mg of total protein.

^c ND, not detected (< 0.001)

Values shown are the mean ± standard deviation for at least three independent biological replicates, with assays done in triplicate. Urease activities measured for all DMG-supplemented conditions are significantly lower compared to the no-DMG control ($p < 0.01\%$, Student's *t*-test).

High levels of DMG are not toxic for mice or wax moth larvae.

While *in vitro* DMG-mediated inhibition of *Salmonella* and *K. pneumoniae* strains appear promising, the relatively high (millimolar) concentrations of DMG required to inhibit these pathogens' growth could impede DMG's *in vivo* use, due to toxicity concerns. A series of preliminary experiments were conducted to evaluate the toxicity of DMG on two animal models, *Mus musculus* (mice) and *Galleria mellonella* (greater wax moth). Mice (BALB/c) have been used (typhoid fever-mouse model) to study *S. Typhimurium* virulence (Maier *et al.* 2014 PLoS One 9:e11018710; Lamichhane-Khadka *et al.* 2015 Infect Immun 83:311-316; Gunn *et al.* 2000 Infect Immun 68:6139-6146), and wax moth larvae have been proven to be a reliable model for studying virulence of many pathogens, including *K. pneumoniae* (Shi *et al.* 2018 BMC Microbiol 18:94; Esposito *et al.* 2018. Front Microbiol 9:1463; Insua *et al.* 2013 Infect Immun 81:3552-3565) and *S. Typhimurium* (Scalfaro *et al.* 2017 FEMS Microbiol Lett 364: 43; Kurstak *et al.* 1968 Can J Microbiol 14:233-237; Bender *et al.* 2013 PLoS One 8:e73287; Viegas *et al.* 2013 Appl Environ Microbiol 79:6124-6133). Mice were subjected to the following DMG treatment: two daily doses of 0.2 mL DMG at 50 mM (~6.1 mg DMG per day), for four consecutive days. These animals displayed no obvious toxicity symptoms (for example, no apparent change in health or behavior compared to the no DMG group control). In another experiment, mice received a daily dose of 0.1 mL 40 mM DMG (~1.2 mg per day) for 4 days, 0.2 mL of 40 mM DMG (~2.4 mg per day) for four days, and then two days of 0.2 mL of 100 mM DMG (~6.1 mg per day). Again, these mice displayed no toxicity symptoms over this course of chelator administration, or for the next three days after cessation of chelator administration (mice were then euthanized). Thus, orally delivered DMG does not appear to be toxic to mice (under the conditions described herein). By comparison, EDTA and its derivatives have been shown to be quite toxic to animals (for a review, see Lanigan *et al.* 2002 Int J Toxicol 21 Suppl 2:95-142): for instance, the acute oral LD₅₀ of Disodium EDTA was found to be 400 mg/kg (Brendel *et al.* 1953 J Am Pharm Assoc Am Pharm Assoc 42:123-124); this corresponds to approximately 8 mg of Na₂-EDTA for mice with an average weight of 20 g. The Ni-chelator disulfiram is even more toxic, with an oral LD₅₀ of disulfiram for mice reported to be as low as 1.013 mg/kg: this corresponds to approximately 20 µg per mouse. Thus, although no formal toxicity study (for example, LD₅₀) was conducted with DMG, these results suggest oral DMG is less toxic in mice than Na₂-EDTA, and far less toxic than disulfiram. The toxicity of DMG was also assessed in wax moth larvae. Injection of 5 µL of increasing concentrations of DMG (ranging from

25 mM to 400 mM) into the rear proleg of *G. mellonella* was not detrimental to the larvae, since 70% to 100% of larvae in each group (n=10 for each group) were still alive 72 hours after injection.

Orally-administered DMG can be detected in mouse livers.

5 Since water soluble DMG had never been used in animals prior to this study, the intestinal absorption and catabolism of orally delivered DMG in mice was unknown. Therefore, Nuclear Magnetic Resonance (NMR) was used to detect the presence of DMG in liver samples of mice that had been given a daily oral dose (6. 1 mg) of aqueous DMG for 2 to 3 days. Although NMR signals from DMG could not be detected in the aqueous supernatant from liver homogenate, diagnostic
10 signals assigned to the methyl carbon (12.0 ppm) and protons (2.04 ppm), and to the oxime carbon (157.8 ppm) were observed in the chloroform extracts of the same supernatant (FIG. 3, Panels A1 and A2) (Shaker *et al.* 2010 Journal of Chemistry 7(S1),S580-S586). In contrast these signals were absent in the chloroform extracts derived from liver samples of the no DMG-control mice (FIG. 3, Panel B). These results strongly suggest that oral DMG is intestinally absorbed and thus can be
15 detected in the liver indicating that orally-delivered chelator has the potential to inhibit pathogens systemically.

Oral delivery of DMG attenuates *Salmonella* virulence in mice.

 The *in vivo* efficacy of DMG against *S. Typhimurium* was assessed in mice, using the
20 mouse-adapted *S. Typhimurium* strain 14028, as previously described (Gunn *et al.* 2000 Infect Immun 68:6139-6146). In this typhoid fever-mouse model, the outcome of oral infection with *S. Typhimurium* is reproducible, typically resulting in a 100% mortality rate within a week (Maier *et al.* 2004. Infect Immun 72:6294-6299; Maier *et al.* 2014. PLoS One 9:e11018; Lamichhane-Khadka *et al.* 2015. Infect Immun 83:311-316). This mortality was confirmed herein: oral infection of mice
25 with 10⁶ bacterial cells (and no DMG) led to 100% mortality within 6 days, in three independent experiments (*see* FIG. 4; N=12 total). To determine the efficacy of DMG, a 3-mg dose of the Ni-chelator was orally delivered to mice 6 hours post-infection and the same treatment (for example, single daily inoculation of 3 mg DMG) was repeated every day for 3 days, 7 days (FIG. 4A) or 9 days (FIG. 4B). The 3-day DMG treatment postponed for 2 days the time of death, however it did
30 not change the final outcome (*e. g.* 100% mortality). By contrast, oral delivery of DMG for 7 days (n=8) and 9 days (n=4) resulted in 37.5% (FIG. 4A) and 50% mouse survival (FIG. 4B), respectively. Thus, oral administration of nontoxic amounts of DMG to *S. Typhimurium*-infected

mice attenuates bacterial virulence, and even leads to host survival (for up to 50% of animals). These results suggest DMG, and by extension Ni-chelation therapy, can be safely used to eradicate *S. Typhimurium* in the mouse model host.

5 An additional experiment was performed to evaluate the effect of DMG on bacterial loads in key organs (liver and spleen) which are typically colonized in the typhoid fever-mouse model. Two groups (n=8 each) of mice were inoculated with *S. Typhimurium* 14028; one group was orally given DMG (3 mg) three times (24 hours and 30 minutes before infection, and 24 hours post infection) while the other group did not receive any DMG. The bacterial burden in livers and spleens was determined three days after infection (FIG. 5). Significantly lower bacterial colonization was found
10 in both livers and spleens of DMG-treated animals compared to the non-DMG treated group. These results confirm the capacity of DMG to inhibit *S. Typhimurium in vivo*, in agreement with the lower death rate observed in DMG-treated animals.

Injection of DMG reduces virulence of MDR enterobacteriaceae in the *Galleria mellonella* insect model.

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Attempts to establish a reliable *K. pneumoniae* infection model in mice failed, so an alternate animal model was chosen. As discussed above, the wax moth (*G. mellonella*) larva model has been already used to study virulence of both *K. pneumoniae* and *S. Typhimurium*, and high levels of DMG (5 μ L of a 400 mM aqueous solution; approximately 0.61 mg) appear to be
20 innocuous, as determined in this study. Injection of larvae with approximately 10^6 CFUs of either *K. pneumoniae* and *S. Typhimurium* resulted in 100% mortality within 16 hours (FIG. 6). In contrast, when DMG (5 μ l of a 250 mM aqueous solution; approx. 0.38 mg) was injected 5 to 10 minutes prior to bacterial challenge, the treatment led to 40% and 60% survival rate for MDR *K. pneumoniae* and MDR *S. Typhimurium*, respectively. These results indicate DMG can attenuate
25 both pathogens *in vivo* in the *G. mellonella* insect model.

In conclusion, the *in vitro* and *in vivo* results presented herein suggest that the growth of Enterobacteriaceae, including that of multi-drug resistant species of *K. pneumoniae* and *S. Typhimurium*, can be inhibited by the Ni-chelator DMG. The observed phenotype could be attributable to inhibition of important Ni-enzymes such as hydrogenase and/or urease. Other effects
30 of DMG on cellular metabolism should not be ruled out, however. While full growth inhibition of the pathogens required moderate to elevated levels of DMG, it is worth noting such levels of Ni-chelator appear to be innocuous when tested on two well defined animal models, including, for

example, mice and wax moth larvae. These results suggest DMG-mediated chelation should be considered an alternate therapy method, especially when dealing with the ever-growing threat of MDR bacterial species.

5 EXPERIMENTAL PROCEDURES

Bacterial strains. The MDR strain of *K. pneumoniae* subsp. *pneumoniae* used in this study was ATCC BAA-2472, a New Delhi metallo-beta-lactamase (NDM-1) positive strain, resistant to aminoglycosides, macrolides, fluoroquinolones, and most β -lactams, including ertapenem. *S. Typhimurium* ATCC 14028 was used for mouse colonization experiments. *S. Typhimurium* ATCC 700408 is a MDR strain, resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides, tetracycline, among others.

Chemicals. Different batches of DMG were used in this study (all from Sigma-Aldrich, St Louis, MO). For *Salmonella* hydrogenase inhibition experiments, DMG D1885 (anhydrous) was used. For growth inhibition experiments, DMG D160105 (disodium salt) was used. For all other experiments, including animal studies, DMG 40400 (disodium salt, octahydrate) was used. (See Table 4.)

Growth conditions. All strains were routinely grown on LB agar plates or in LB broth. For DMG-growth inhibition study, *S. Typhimurium* strains ATCC 14028 and ATCC 700408 were grown in M9 minimal medium (M9 minimal salts, 0.4% glucose, 2 mM MgSO_4 , 0.1 mM CaCl_2 , and 1 $\mu\text{g/mL}$ thiamine, pH 7.2). *K. pneumoniae* was grown in “W-U” medium, modified from Bender *et al.* 1977 129:1001-1009, containing 60 mM K_2HPO_4 , 33 mM KH_2PO_4 , 0.4 mM MgSO_4 , 0.4% Glucose, and 5 mM urea (instead of KNO_3), pH 7.4. Briefly, overnight cultures of *S. Typhimurium* and *K. pneumoniae* were harvested, spun at 14,000 rpm for 5 minutes, washed once with either M9 (*S. Typhimurium*) or W-U (*K. pneumoniae*) and resuspended in either M9 (*S. Typhimurium*) or W-U (*K. pneumoniae*). Cells were inoculated to an OD_{600} of 0.005 in M9 or W-U media, in presence of increasing concentrations of DMG (2.5 mM, 5 mM, 7.5 mM, or 10 mM). Cells were incubated for 24 hours at 37°C with shaking at 250 rpm, then serially diluted in PBS and 5 μL of each dilution was spotted in triplicate on LB plate. Colony forming units (CFU) were counted after 16 hours at 37°C. Each growth experiment was done at least 3 times.

Amperometric hydrogenase assays. The hydrogenase activity of *S. Typhimurium* ATCC strain 14028 was assayed by using an amperometric method, as previously described (Lamichhane-Khadka *et al.* 2015 Infect Immun 83:311-316). Briefly, cells were grown on blood agar media for

6 hours under a H₂-enriched microaerobic atmosphere (Maier *et al.* 2004 Infect Immun 72:6294-6299), supplemented with only DMG (0.1 mM, 0.5 mM, 1 mM, 5 mM, or 10 mM) or 1 mM DMG and 50 µM NiCl₂. Cells were suspended in phosphate-buffered saline (PBS) to a final concentration of 8×10^8 per mL and added to a sealed amperometric dual-electrode chamber, with constant stirring; 100 µL of H₂-saturated phosphate-buffered saline was added to the chamber and the disappearance of H₂ was recorded over time. Hydrogenase activity is expressed in nmoles of H₂ oxidized per minute per 10^8 cells.

Urease assays. *K. pneumoniae* cells were grown overnight at 37°C in LB (control) or LB supplemented with 1, 2, or 5 mM DMG at 37°C with shaking at 200 rpm. Cells were pelleted at 6,000 rpm for 30 minutes and washed three times with PBS (pH 7.4). Cells were standardized to an optical density (OD₆₀₀) of 4.5, added to sterile glass beads (0.1 mm diameter, Biospec Products, Bartlesville, OK) at a 100% w/v ratio, and frozen at -80°C. The bead and cell mixture was thawed at room temperature and vortexed at 3,200 rpm for 6 minutes with 1 minute intervals on ice. The mixture was pelleted at 14,000 rpm for 2 minutes and the cell-free supernatant was assayed for urease activity using the phenol-hypochlorite method (Weatherburn *et al.* 1967. Analytical Chemistry 39:971-974). Protein concentration was determined using the BCA protein kit (Thermo Fisher Pierce, Rockford, IL, USA). Urease activity is expressed as µmoles of NH₃ produced per minute per mg of total protein.

Mouse experiments – Chelator toxicity. A group of 8 mice were subjected to the following DMG treatment: two daily doses of 0.2 mL DMG at 50 mM (~6.1 mg DMG per day) for four consecutive days. These animals displayed no obvious toxicity symptoms. In another experiment, mice received a daily dose of 0.1 mL 40 mM DMG (~1.2 mg per day) for 4 days, 0.2 mL of 40 mM DMG (~2.4 mg per day) for four days, and then 0.2 mL of 100 mM DMG (~6.1 mg per day) for two days. Again, these mice displayed no toxicity symptoms over this course of chelator administration, or for the next three days after cessation of chelator administration (mice were then euthanized).

Mouse experiments – Detection of DMG in liver samples. A group of 8 mice was used for this experiment: 2 mice were given 0.2 mL of 100 mM DMG (~6.1 mg per day) for two days and then euthanized, and 2 mice were given the same dose for three days and then euthanized; the remaining 4 mice were used as no DMG-control. Mice were sacrificed by CO₂ asphyxiation and cervical dislocation. Livers were quickly removed and homogenized in 2 mL sterile deionized water using a tissue homogenizer (“Tissue Tearor” model 985370, Biospec products, Bartlesville, OK,

USA). Homogenized liver samples were spun at 16,800 x g for 6 min, and supernatants were collected before being passaged through a 0.45 µm filter unit. Filtered supernatants were subjected to NMR analysis. Since preliminary NMR experiments failed to detect DMG in individual liver samples from DMG-treated mice, these four samples were pooled, concentrated and also extracted with chloroform for additional NMR analysis. Liver samples from no-DMG treated mice were similarly processed and used as negative controls.

Mouse experiments – Infection Experiments. The *in vivo* efficacy of DMG against *S. Typhimurium* was assessed by using the typhoid fever-mouse model, as previously described (Gunn *et al.* 2000 Infect Immun 68:6139-6146). Female BALB/c mice (Charles River, Boston, MA) were orally inoculated individually with the *S. Typhimurium* strain ATCC 14028, following previously described methods (Maier *et al.* 2004 Infect Immun 72:6294-6299; Maier *et al.* 2014 PLoS One 9:e110187; Lamichhane-Khadka *et al.* 2015 Infect Immun 83:311-316). Briefly, *S. Typhimurium* cells grown overnight in LB were harvested, washed and suspended in sterile PBS to a final OD of OD₆₀₀ 0.01 (approximately 10⁷ CFU/mL) and 0.1-mL volumes (10⁶ bacterial cells) were introduced orally into each mouse. A dose of 3 mg of DMG, corresponding to either 0.1 mL of a 100 mM or 0.2 mL of a 50 mM DMG aqueous solution, respectively, was orally given to mice belonging to one group, while the other group of mice (control) was only given sterile H₂O. The DMG treatment was performed 6 hours post-infection, then (once) every 24 hours post-infection, for 3 to 9 days, as described for each experiment (three independent experiments, with n=4 to n=8 mice for each experiment). The mice were observed twice daily, and morbidity was recorded. In addition, a fourth independent experiment was performed to determine organ (liver and spleen) bacterial burdens. In this experiment, two groups of 8 mice each were inoculated with *S. Typhimurium* 14028. One group was treated for 3 days with one daily dose of DMG (3 mg), at 24 hours and 0.5 hours before infection, and 24 hours post infection. Infection with *S.T.* 14028 was done as described above. Mice were euthanized 72 hours after infection. Livers and spleens were removed and homogenized in sterile PBS. Dilutions of the homogenate were plated on bismuth sulfite agar plates (BD DIFCO, Becton Dickinson, Franklin Lakes, NJ), a selective medium for *Salmonella* species. Colony forming units (CFU) were counted after overnight incubation of the plates at 37°C.

***Galleria mellonella* (wax moth) experiments.** Wax moth larvae were obtained from local pet stores, from two different suppliers: the bug company (Ham Lake, MN; www.ewbugco.com) and Timberline (Marion, IL; www.timberlinefresh.com). Larvae were stored in wood shavings at 4°C in the dark and used within 2 weeks after purchase. Only larvae weighing 300 ± 50 mg were selected

for the experiments. Groups of 10 larvae were used for each experiment. The site of injection (last right or left proleg) was disinfected with ethanol 70% (vol/vol) before and after each injection. A 10 μ L-Hamilton syringe fitted with a 30.5-gauge needle (Becton Dickinson, Franklin Lakes, NJ) was used to inject 5 μ L. After injection, larvae were kept in 9.2-cm Petri dishes at 37°C in the dark with no food. Larvae were monitored daily for up to 4 days and mortality (as defined by change of pigmentation and absence of response following physical stimulation) was recorded. **Chelator toxicity:** the following concentrations of DMG were tested (n=10 for each): 5, 10, 25, 50, 75, 100, 125, 150, 200, 250, 300, 350, 400 mM. Each solution was freshly prepared in 0.8 % sterile NaCl, and 5 μ L of each solution was injected in the last right proleg. A control with no chelator (0.8% NaCl only) was included in the study. **Infection experiment:** 5 μ L of DMG (250 mM) was injected in the last right proleg of each larva, then approximately 5×10^5 bacteria (either MDR *K.p.*, or MDR *S.T.*) were injected in the last left proleg 5 to 10 minutes after DMG inoculation. Bacterial suspensions were prepared as follows: cells were grown overnight in 5 mL of Mueller Hinton broth, harvested, washed once with and resuspended in sterile 0.8% NaCl to a final OD₆₀₀ of 0.1 (approximately 10^8 CFU/mL, as determined by CFU counts on serially diluted samples). Death rates were compared to those obtained after injection of NaCl only, DMG (250 mM) only, MDR *K.p.* only, or MDR *S.T.* only, respectively.

Nuclear magnetic resonance. All data were collected on a Bruker Avance Neo 800 MHz equipped with a 1.7 mM cryoprobe at 25°C. Standard proton spectra (without and with water suppression) and two-dimensional one-bond and multiple-bond proton-carbon correlated spectra (HSQC and HMBC) were collected using Bruker pulse library sequences, zg, zgpr, hsqcetgpcp2.2, and hmbcetgpl3nd, respectively. Data were processed with Mnova software (Mestrelab Research, Spain). Initial NMR samples were prepared from aqueous liver, spleen and blood preparations of individual mice, by adding 5 μ L of D₂O to a 50 μ L aliquot from each sample. Since the HSQC spectrum showed no detectable DMG, the two sets of liver samples (with DMG, no DMG) were then separately pooled, lyophilized and 100 μ L D₂O was added to the residue. Once again, no DMG was detected in the aqueous sample. The two concentrated liver DMG samples were then extracted twice with 1 mL chloroform. The organic phase was separated and dried. 50 μ L of CDCl₃ was added to the residue and used for NMR analysis (see FIG. 3). NMR assignments were based on similar compounds described in Shaker *et al.* (2010 Journal of Chemistry 7(S1),S580-S586) and were confirmed using authentic aqueous samples of DMG and DMG + NiCl₂ and their chloroform extracts.

Table 4.

Reference	Label (Manufacturer)	Solubility	Chemical formula	FW (g/mol)	Concentration used		Vol. used	Quantity compd (mg)	DMG only (mg)	Experiment/ Application
					mM	mg/mL				
D-1885	Dimethylglyoxime	Non water soluble	$C_4H_8N_2O_2$	116.12	0.1-10	0.011-0.11	NA	NA	NA	S.T. hydrogenase
	(ALDRICH)	50 mg/mL in warm ethanol								inhibition
D-160105	Dimethylglyoxime,	High Water solubility	$C_4H_8N_2O_2$	160.1	2.5-20 mM	0.4-3.2	NA	NA	NA	Bacterial (Hp, Kp, ST)
	disodium salt hydrate, 97%	~ 190 g/L or ~ 1.2 M (SB)	2Na							growth inhibition
40400	(SIGMA-ALDRICH)									
	Dimethylglyoxime,	High water solubility	$C_4H_8N_2O_2$	304.2	50 mM	15.2	0.2 mL	3.04 mg	1.16 mg	Mice/ ST inhibition
	disodium salt octahydrate, > 97%	~ 366 g/L or ~ 1.2 M (SB)	2Na, 8 H ₂ O		100 mM	30.4	0.1 mL	3.04 mg	1.16 mg	Mice/ ST inhibition
					100 mM	30.4	0.2 mL	6.08 mg	2.32 mg	Mice/NMR
					200 mM	60.8	0.1 mL	6.08 mg	2.32 mg	Mice/NMR
	(FLUKA-SIGMA-ALDRICH)				25-125 mM	7.6-38.1	5 µL	38-190 µg	14-72 µg	Wax moth larvae
					2.5-10 mM	0.76-3.0	NA	NA	NA	Kp urease inhibition
					2.5-20 mM	0.76-6.1	NA	NA	NA	Growth inhibition (Kp,

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Example 2 – Effect of Metals and DMG-Chelation on β -amyloid peptides

In this Example, the aggregation of β -amyloid peptide in the presence or absence of different metals and/or DMG was measured.

5 The aggregation of β -amyloid (1-40, human) peptide was monitored by fluorescence: the fluorophore (thioflavin) binds in the groove along the surface of the β -amyloid peptide fibers. Formation of fibers (aggregation) leads to increased fluorescence.

Example 2A

10 In a first experiment, fluorescence/aggregation of 100 μ M β -amyloid (1-40) peptide was followed for 2 hours, with readings taken every 15 minutes (following 10 seconds shaking). The experimental conditions are shown in Table 5. Results are shown in Table 6 and FIG. 7.

15 Aggregation of β -amyloid (1-40) peptide was observed in the absence of any metal, and addition of Copper (Cu), Nickel (Ni) and Zinc (Zn) accelerated/increased aggregation with 100 μ M β -amyloid (1-40) peptide in the presence of the fluorophore (thioflavin) and nickel, copper, or zinc exhibiting very early saturation (see Table 6).

Example 2B

20 In a second experiment, fluorescence/aggregation of 50 μ M β -amyloid (1-40) peptide was followed for 1 hour, with readings taken every 5 minutes. The experimental conditions are shown in Table 7. Results are shown in FIG. 8.

Metals were observed to enhance aggregation, with the effects descending as follows: Zn>Ni>Mn>Cu>Co>Fe. In the absence of added metals, DMG was observed to slow down (at 100 μ M DMG) or inhibit (at 1 mM DMG) β -amyloid (1-40) peptide aggregation.

25 When DMG was added in the presence of nickel, the effect of DMG was found to depend on the ratio of DMG to nickel. At a 10:1 DMG:nickel ratio, inhibition was observed, but no inhibition was observed when using equimolar concentrations (1:1) of DMG and Ni (100 μ M each). These results are consistent with the observation that 2 molecules (or moles) of DMG are needed to chelate 1 molecule (mole) of Ni (FIG. 1A).

30

Example 2C

In a third experiment, fluorescence/aggregation of previously frozen 50 μ M β -amyloid (1-40) peptide was followed for 2 hours, with readings taken every 5 minutes. The experimental conditions were as shown in Table 7 with additional metals tested in combination with DMG.

- 5 Results are shown in FIG. 9. DMG was observed to partially inhibits copper- and nickel-mediated β -amyloid (1-40) peptide aggregation at a 10:1 DMG:metal ratio; however, no inhibition was observed at this same ratio with zinc, confirming DMG specificity towards nickel (and, to a lesser extent, copper).

Table 5. Components and volumes used for β -amyloid aggregation inhibition experiment.

NiCl₂: Nickel chloride; CuSO₄: copper sulfate; ZnSO₄: zinc sulfate; DMG: dimethyl glyoxime; β -A: beta-amyloid 1-40 peptide; TF: thioflavin.

#	Name	Water	NiCl ₂ (10 mM)	CuSO ₄ (10 mM)	ZnSO ₄ (10 mM)	DMG (10 mM)	β -A (1.25 x)	TF (400 μ M)
1	Neg C	90					0	10
2	Pos C	10	0	0	0	0	80	10
3	Ni	0	10	0	0	0	80	10
4	Cu	0	0	10	0	0	80	10
5	Zn	0	0	0	10		80	10
6	DMG	0	0	0	0	10	80	10

Table 6. Effect of metal or DMG-Chelation on aggregation of β -amyloid peptide, as measured using fluorescence
 TF: Thioflavin; A: β -amyloid 1-40 peptide; Ni: Nickel sulfate; Cu: Copper sulfate; Zn: Zinc sulfate; DMG: dimethyl glyoxime, chelator.

Time (min)	TF only		TF, A 100 μ M		TF, A 100 μ M, Ni 1 mM		TF, A 100 μ M, Cu 1 mM		TF, A 100 μ M, Zn 1 mM		TF, A 100 μ M, DMG 1 mM	
	B4	B5	C4	C5	D4	D5	E4	E5	F4	F5	G4	G5
0	13,807	13,655	55,362	56,845	Saturation	73,858	57,435	66,884	Saturation	Saturation	80,887	51,850
10	14,031	14,029	59,950	61,926	Saturation	Saturation	62,143	79,936	Saturation	Saturation	83,040	50,591
20	14,114	13,961	67,048	70,131	Saturation	Saturation	69,499	90,940	Saturation	Saturation	87,361	52,171
30	14,096	13,917	71,911	74,644	Saturation	Saturation	84,288	97,369	Saturation	Saturation	90,927	54,676
40	13,522	13,647	76,209	79,522	Saturation	70,868	83,298	Saturation	Saturation	Saturation	89,749	55,009
50	13,523	13,519	81,042	84,741	Saturation	59,623	83,983	Saturation	Saturation	Saturation	84,647	57,496
60	13,579	13,379	85,775	89,151	Saturation	Saturation	85,286	Saturation	Saturation	Saturation	81,927	57,615
70	13,534	13,302	89,875	94,318	Saturation	Saturation	87,481	Saturation	Saturation	Saturation	78,665	54,784
80	13,417	13,289	94,407	98,240	Saturation	Saturation	89,319	Saturation	Saturation	Saturation	76,494	54,814
90	13,389	13,296	98,771	Saturation	Saturation	Saturation	90,629	Saturation	Saturation	Saturation	70,324	54,502
100	13,225	13,097	Saturation	Saturation	Saturation	Saturation	92,983	Saturation	Saturation	Saturation	71,298	54,188
110	13,395	13,101	Saturation	Saturation	Saturation	Saturation	95,241	Saturation	Saturation	Saturation	75,279	54,232
120	13,141	12,943	Saturation	Saturation	Saturation	Saturation	97,368	Saturation	Saturation	Saturation	76,734	55,058
130	13,046	12,998	Saturation	Saturation	Saturation	Saturation	99,459	Saturation	Saturation	Saturation	76,441	57,009
140	13,104	12,992	Saturation	Saturation	Saturation	93,783	Saturation	Saturation	Saturation	Saturation	74,893	54,009
150	13,003	12,750	Saturation	Saturation	Saturation	Saturation	Saturation	Saturation	Saturation	Saturation	69,683	54,304
160	12,883	12,857	Saturation	Saturation	Saturation	Saturation	Saturation	Saturation	Saturation	Saturation	63,919	54,878
170	12,771	13,003	Saturation	Saturation	Saturation	78,001	Saturation	Saturation	Saturation	Saturation	62,412	54,166
180	12,815	12,732	Saturation	Saturation	Saturation	87,983	Saturation	Saturation	Saturation	Saturation	66,421	54,239

Table 7. Components and volumes used for β -amyloid aggregation inhibition experiment.

TBS: Tris Buffer Saline; CoCl_2 : Cobalt chloride; CuSO_4 : Copper sulfate; FeSO_4 : Iron sulfate; MnSO_4 : Manganese sulfate; NiCl_2 : Nickel chloride; CuSO_4 : copper sulfate; ZnSO_4 : zinc sulfate; DMG: dimethyl glyoxime; β -A: beta-amyloid 1-40 peptide.

1) Reaction mixes All in quadruplicate wells

#	B3-B5	B6-B8	C3-C5	C6-C8	D3-D5	D6-D8	E3-E5	E6-E8	F3-F5	F6-F8	G3-G5	G6-G8	Final conc (uM)
Name	Neg C no BA	Pos C with BA	Co	Cu	Fe	Mn	Ni	Zn	DMG 0.1	DMG 1	Ni-DMG 0.1	Ni-DMG 1	
Water	80	60	50	50	50	50	50	50	50	50	40	40	NA
TBS 10 x	10	10	10	10	10	10	10	10	10	10	10	10	1X
CoCl_2 (1 mM)	0	0	10	0	0	0	0	0	0	0	0	0	100
CuSO_4 (1 mM)	0	0	0	10	0	0	0	0	0	0	0	0	100
FeSO_4 (1 mM)	0	0	0	0	10	0	0	0	0	0	0	0	100
MnSO_4 (1 mM)	0	0	0	0		10	0	0	0	0	0	0	100
NiCl_2 (1 mM)	0	0	0	0	0	0	10	0	0	0	10	10	100
ZnSO_4 (1 mM)	0	0	0	0	0	0	0	10	0	0	0	0	100
DMG (1 mM)	0	0	0	0	0	0	0	0	10	0	10	0	100
DMG (10 mM)	0	0	0	0	0	0	0	0	0	10	0	10	1000
Thioflavin (400 uM)	10	10	10	10	10	10	10	10	10	10	10	10	40
β -A mix (250 uM)	0	20	20	20	20	20	20	20	20	20	20	20	50

2) Peptide solubilization (prepare last, and add last in 96 well plate)

Component	MW (Da or g/mol)	Amount (mg)	Vol. (NaOH)		Stock Concentration	
			(uL)		(uM)	
Beta-amyloid 1-40	4330	1	924		1.08	
					250	

Example 3 – Dimethylglyoxime inhibition of biofilms

METHODS

Growth conditions. *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) ATCC 700408 and *Klebsiella pneumoniae* ATCC BAA2472 were grown overnight in Luria-Bertani (LB) broth aerobically at 37°C with shaking. *Helicobacter pylori* 43504 was grown on Brucella agar plates supplemented with 10% defibrinated sheep blood (BA) under microaerophilic conditions (4% O₂, 10% CO₂, and 86% N₂) at 37°C.

Biofilm inhibition assay. *S. Typhimurium* and *K. pneumoniae* cultures were adjusted to an optical density (OD₆₀₀) of 0.1 in fresh LB broth. *H. pylori* culture was adjusted to an OD₆₀₀ of 0.15 in brain heart infusion (BHI) supplemented with 0.4% β-cyclodextran. *S. Typhimurium*, *K. pneumoniae*, and *H. pylori* cultures at the above cell densities were added to non-tissue culture treated polystyrene 96 well plates (Falcon) along with dimethylglyoxime disodium salt hydrate (Na₂DMG) at various concentrations. Plates containing *S. Typhimurium* and *K. pneumoniae* were incubated at 37°C aerobically without shaking for 18 hours. Plates containing *H. pylori* were incubated at 37°C microaerobically without shaking for 48 hours. After incubation with (Na₂DMG) a crystal violet biofilm assay was performed as previously described with modifications (Kwasny et al. 2010. Current Protocols in Pharmacology 50:13A. 8.1-13A. 8.23). Briefly, supernatant was removed from the wells. Next, adherent cells were gently washed with phosphate-buffered saline (PBS) and allowed to air dry for 15 minutes. Cells were then heat fixed for 1 hour at 60°C before staining with 0.5% crystal violet for 5 minutes. After the crystal violet was thoroughly washed from the wells with distilled deionized water, cells were air dried for 15 minutes. Acetic acid (33%) was then added to the wells to solubilize the crystal violet. The crystal violet-acetic acid solution was transferred to a clean 96 well plate and the OD₆₀₀ was measured.

Antibiofilm assay. *H. pylori* culture at OD₆₀₀ = 0.15 was added to a non-tissue culture treated polystyrene 96 well plate (Falcon) and incubated at 37°C micro-aerobically without shaking for 48 hours. After the initial incubation, the supernatant was removed from the wells and BHI + 0.4% β-cyclodextran was added back to the wells with or without Na₂DMG at various concentrations. The plate was then incubated an additional 24 hours. Then, the crystal violet biofilm assay was performed as above.

RESULTS

In all three species tested (*S. Typhimurium*, *K. pneumoniae*, and *H. pylori*), DMG was able to inhibit biofilm formation (FIG. 10). Additionally, DMG was able to counteract an established (48 hours of growth) *H. pylori* biofilm (FIG. 11). These experiments provide evidence for promising antibiofilm properties of DMG.

Example 4- Effect of DMG (alone or in combination with metal ions) on *Campylobacter* species.

This Example shows that DMG (at millimolar levels) is bactericidal against *Campylobacter concisus*. This Example further shows that while DMG (at millimolar levels) is bacteriostatic against *Campylobacter jejuni*, DMG at millimolar levels is bactericidal against *C. jejuni* when combined with micromolar levels of copper (Cu^{2+}).

C. concisus has been found throughout the entire human oral-gastrointestinal tract. The bacterium is associated with various ailments and diseases, such as gingivitis, periodontitis, inflammatory bowel disease, including Crohn's disease. There is no known animal reservoir.

C. jejuni is one of the most common causes of food poisoning in Europe and in the United States. The CDC estimates a total of 1.5 million infections every year and the European Food Safety Authority estimates approximately nine million cases of human campylobacteriosis per year in the European Union. People are usually infected by eating raw or undercooked poultry, or eating something that touched the raw or undercooked poultry. People may also be infected by eating seafood, meat, and produce; by contact with animals (*C. jejuni* is commonly found in animal feces); or by drinking untreated water. Food poisoning caused by *Campylobacter* species can be severely debilitating, however it is rarely life-threatening. Nevertheless, it can subsequently lead to Guillain-Barré syndrome (GBS), an auto-immune disease targeting the nerves and eventually causing paralysis.

Fluoroquinolone-resistant *Campylobacter* species are classified as “Priority 2: high” in the WHO’s list of MDR pathogens (see Table 1A). All *Campylobacter* species have at least one Ni-containing hydrogenase (for instance *C. jejuni*), and some have two (for instance *C. concisus*). In the case of *C. concisus*, one of the hydrogenase complexes has been shown to be essential (Benoit et al. 2018 Sci. Rep. 8(1):14203). Hence, inhibiting the Ni-containing catalytic site of *C. concisus*

(with DMG) was expected to have inhibitory effects on *C. concisus* growth and survival. As further described, below, however, the combined effect of DMG and Cu²⁺ on *C. jejuni* was unexpected.

METHODS

Growth conditions. *C. concisus* (strain 13826, ATCC BAA-1457) was routinely grown on Brucella agar supplemented with 10% defibrinated sheep blood (BA plates) or Brain Heart Infusion (BHI) plates supplemented with 10% fetal calf serum (FCS), under H₂-enriched microaerobic conditions (10% H₂, 5% CO₂, 2-10% O₂, balance N₂) at 37°C. *C. jejuni* (strain 81-176, ATCC BAA-2151) was either grown on BA plates or on BHI plates under microaerobic conditions (10% CO₂, 4% O₂, 86% N₂) at 37°C. DMG (sodium salt, octahydrate) was used by itself or in combination with various divalent cations (Cobalt (II), CoCl₂; Copper(II), CuSO₄; Manganese(II), MnSO₄; Nickel(II), NiSO₄; Zinc(II), ZnSO₄)

Growth inhibition assays: solid (Petri dish) plates experiments. *C. concisus* or *C. jejuni* cells were grown as described above, harvested and resuspended in sterile NaCl (8 g/L) to an optical density at 600 nm (OD₆₀₀) of 0.1 or 1; an OD₆₀₀ of 1 corresponds approximately to 1 x 10⁹ cells/mL to 5 x 10⁹ cells/mL (for *C. concisus*) and 5 x 10⁹ cells/mL to 1 x 10¹⁰ cells/mL (for *C. jejuni*). Cells were serially (10-fold) diluted from 10⁻¹ to 10⁻⁶ or 10⁻⁷ in NaCl (8 g/L) and 5 µL of each dilution was spotted on BA plates (for *C. concisus*) or BHI plates (for *C. jejuni*), containing various concentrations of DMG (0.5, 1, 2.5 or 5 mM), with or without various added metals (CoCl₂, CuSO₄, MnSO₄, NiSO₄, ZnSO₄) at the following final concentrations: 1 µM, 5 µM, 10 µM, 20 µM, 50 µM, 100 µM, 500 µM. Plates with metals only were also used as controls. Colony-forming units (CFUs) were counted after 24 hours of growth at 37°C, under atmospheric conditions described above. The number of CFUs for each (DMG and metal) condition was compared to the number of CFUs obtained for the control condition (defined as plain BA, plain BHI-FCS, or plain BHI medium, without any DMG or added metal). Also, the size of the colonies were monitored. DMG concentrations were considered bacteriostatic when the number of CFUs was equal to the number of CFUs of the control, but the size of each CFU was significantly smaller compared to the control. DMG concentrations were considered bactericidal when the number of CFUs was at least three logs less than the number of CFUs obtained for the control.

Growth inhibition assays: liquid (96-well) plates experiments. This type of experiment can only be conducted with *C. jejuni*, since the gas requirement for *C. concisus* (H₂-enriched microaerobic) is not compatible with the use of 96-well plates. *C. jejuni* cells were grown as

described above, harvested and resuspended in sterile NaCl (8 g/L) to an OD₆₀₀ of 0.1, corresponding approximately to 5×10^8 to 1×10^9 cells/mL. A checkboard type of loading plan allows for screening of up to 8 DMG concentrations (0 mM, 0.062 mM, 0.125 mM, 0.25 mM, 0.5 mM, 1 mM, 2 mM, and 4 mM) against 11 metal ions concentrations (0 μ M, 2 μ M, 4 μ M, 8 μ M, 16 μ M, 32 μ M, 64 μ M, 128 μ M, 256 μ M, 512 μ M, and 1024 μ M). Wells were serially (2-fold) diluted in each direction (horizontal and vertical) to introduce all combinations. Wells with no DMG or metal compounds served as (positive) growth controls, and wells with no bacteria served as background (negative growth) controls. A typical protocol and loading plan is described in **Table 8**. The starting inoculum was at OD₆₀₀ of 0.05, which corresponds approximately to 2.5×10^8 cells/mL to 5×10^8 cells/mL. After 24 hours incubation under microaerobic conditions at 37°C, OD₆₀₀ was recorded using a 96 well-plate reader (Microtek). The minimal inhibitory concentration (MIC) for DMG (or metal) is defined as the lowest concentration that inhibits cell growth (for example, OD₆₀₀ at T=24 hours is the same or lower than 0.05) The minimal bactericidal concentration (MBC) was determined as follows: at the end of the MIC assay (24 hours), 5 μ L of each well showing no or low growth was spotted on BHI and incubated at 37°C overnight. Resulting growth (or lack of growth) was examined after overnight culturing; the lowest concentration that inhibits 99.9% of the original culture was defined as MBC. MIC and MBC were determined based on tests (plates) done in triplicate.

RESULTS

Effect of DMG on *C. concisus*: millimolar levels of DMG are bactericidal.

***C. concisus* recoveries on solid plates.** After dilution and spotting on BA plates with no DMG or 2 mM DMG or 4 mM DMG, cells were incubated under H₂-enriched microaerobic conditions for 24 hours at 37°C and CFUs were counted (see **FIG. 12A**, for an example). The number of CFUs on BA plates supplemented with DMG 2 mM were equal or slightly lower than the number of CFUs on the control plates (no DMG), however the size of the colonies was smaller, suggesting the DMG concentration (2 mM) is bacteriostatic. There was no CFU detected on BA supplemented with DMG 4 mM (detection limit: 200 CFUs), indicating DMG at 4 mM is bactericidal for *C. concisus* under these experimental conditions.

Effect of DMG on *C. jejuni*: millimolar levels of DMG are bacteriostatic, but addition of micromolar levels of copper (II) renders millimolar levels of DMG bactericidal towards *C. jejuni*.

C. jejuni recoveries on solid plates. After dilution and spotting on BHI plates with no DMG, or various concentrations of DMG with or without supplemental CuSO₄ or other metals (see methods), cells were incubated under microaerobic conditions for 24 hours at 37°C and CFUs were counted (see **Fig. 8B**). The number of CFUs on BHI plates supplemented with 5 mM DMG were equal or slightly lower than the number of CFUs on the control plates (having no DMG), however the size of the colonies was smaller, suggesting the DMG concentration (5 mM) is bacteriostatic. When copper (II) (CuSO₄) at the following final concentrations: 1 µM, 5 µM, 10 µM, 20 µM, 50 µM, 100 µM, 500 µM) was added to 5 mM DMG plates, there was no detectable growth on the plates (detection limit: 200 CFUs), indicating all of the above combinations were bactericidal for *C. concisus* under these experimental conditions (see **Fig. 8B**).

The bactericidal effect of the DMG/metal combination is specific to Cu(II). For instance, while a DMG (2 mM)/Cu (0.5 mM) combination is bactericidal, there was no noticeable effect on *C. jejuni* growth when 0.5 mM of CoCl₂, MnSO₄, NiSO₄, or ZnSO₄ was added to the medium in combination with 2 mM DMG.

C. jejuni liquid (96-well checkboard assays) growth inhibition experiments. After 24 hours incubation under microaerobic conditions at 37°C, the OD₆₀₀ was recorded in each well using a 96 well-plate reader. **Table 9** shows an example of results obtained with various DMG and CuSO₄ concentrations (three plates combined). In this case, the minimal inhibitory concentration (MIC), as defined in the Methods section, corresponds to the following combinations of DMG (in mM) and CuSO₄ (in µM): 4 and 8; 2 and 64; 1 and 256 (see **Table 10**).

After the OD₆₀₀ was recorded, 5 µL of each well showing no or low growth was spotted on BHI and incubated at 37°C overnight to determine the minimal bactericidal concentration (MBC), as defined in the Methods section.

As observed with the solid plate experiment, the bactericidal effect of DMG/metal combination is specific to Cu(II). Other metals did not confer the same potency in combination with DMG, therefore the DMG/Cu combination is the most efficient (*e.g.* bactericidal) against *C. jejuni*. **Table 10** summarizes the MIC/MBC obtained for *C. jejuni*, with DMG and either CoCl₂, MnSO₄, NiSO₄, or ZnSO₄.

Table 8. *C. jejuni* 81-176 against DMG and CuSO₄, checkerboard strategy.

Final DMG (mM)		Final CuSO ₄ concentration (uM)											
		0	2	4	8	16	32	64	128	256	512	1024	1024
4	A	1	2	3	4	5	6	7	8	9	10	11	12
2	B	no Cu											no cell
1	C	no Cu											no cell
0.5	D	no Cu											no cell
0.25	E	no Cu											no cell
0.125	F	no Cu											no cell
0.062	G	no Cu											no cell
0	H	no Cu/ no DMG	no DMG	no DMG	no DMG	no DMG	no DMG	no DMG	no DMG	no DMG	no DMG	no DMG	Blank
1) Add 100 uL of MH everywhere (A1-H12)													
2) Add 100 uL of 16 mM DMG (240 uL 200 mM DMG.8H2O in 2760 uL MH) in rows A1-A12													
3) Serially (2-fold) dilute DMG from row A (A1-A12) to row G (G1-G12). Discard 100 uL after row G.													
4) Volume should be 100 uL everywhere and DMG concentration should be 8 mM (row A) to 0.125 mM (row G), no DMG in row H													
5) Add 100 uL of 4.096 mM CuSO ₄ (61.5 uL 200 mM CuSO ₄ in 3 mL MH) in columns 11 (A11-H11) and 12 (A12-H12). Column 12: no cell control (metal-only OD ₆₀₀)													
6) Serially (2-fold) dilute metal from column 11 (A11-H11) to column 2 (A2-H2). Discard 100 uL after column 2.													
7) Volume: 100 uL everywhere, except in 12 (200 uL); CuSO ₄ concentration: 2048 uM (Column 12) to 2 uM (Column 2), no Cu in column 1.													
8) Add 100 uL of bacteria (<i>C. jejuni</i>) at OD 0.1 everywhere except in column 12. Starting OD should be 0.05 everywhere.													
9) Final concentrations: DMG concentration range from 4 to 0.062 mM, CuSO ₄ conc. range from 1.024 mM to 0.002 mM (1,024 uM to 2 uM)													
10) Incubate <i>C. jejuni</i> in CO ₂ incubator (4% O ₂ , 10% CO ₂ , balance N ₂) for 24 h													
11) Measure OD ₆₀₀ at 24h. Blank with A12. Use Column 12 to withdraw Cu-and DMG-Cu absorbance.													

Table 9.

3 plates combined		Final CuSO ₄ concentration (uM)										No cell
Final	0	2	4	8	16	32	64	128	256	512	1024	1024
DMG (mM)	1	2	3	4	5	6	7	8	9	10	11	12
4	A	0.19	0.26	0.15	0.04	0.00	0.01	0.01	0.02	0.02	0.04	0.04
2	B	0.24	0.34	0.25	0.26	0.17	0.01	0.01	0.01	0.02	0.04	0.04
1	C	0.20	0.27	0.23	0.21	0.24	0.19	0.10	0.02	0.02	0.04	0.04
0.5	D	0.22	0.31	0.25	0.23	0.21	0.19	0.20	0.29	0.14	0.09	0.04
0.25	E	0.26	0.31	0.24	0.37	0.25	0.22	0.24	0.24	0.27	0.30	0.04
0.125	F	0.25	0.31	0.27	0.31	0.26	0.23	0.24	0.27	0.34	0.47	0.04
0.062	G	0.31	0.37	0.31	0.33	0.24	0.22	0.24	0.35	0.34	0.47	0.04
0	H	0.23	0.29	0.23	0.31	0.26	0.27	0.23	0.38	0.28	0.43	0.04

Table 10. MIC/MBC for DMG-metal combinations, *C. jejuni* strain 81-176

DMG concentration (mM)					
Metal	0	0.5	1	2	4
Co ²⁺	>1024 / >1024	>1024 / >1024	>1024 / >1024	512 / 1024	256 / 512
Cu ²⁺	>1024 / >1024	1024 / >1024	256 / 512	64 / 128	8 / 16
Ni ²⁺	>1024 / >1024	>1024 / >1024	>1024 / >1024	>1024 / >1024	>1024 / >1024
Zn ²⁺	512 / >1024	512 / >1024	512 / >1024	512 / >1024	512 / >1024

First number, bold: MIC (uM) / second number, italic: MBC (uM)

The complete disclosure of all patents, patent applications, and publications, and electronically available material (including, for instance, nucleotide sequence submissions in, for example, GenBank and RefSeq, and amino acid sequence submissions in, for example, SwissProt, PIR, PRF, PDB, and translations from annotated coding regions in GenBank and RefSeq) cited
5 herein are incorporated by reference. In the event that any inconsistency exists between the disclosure of the present application and the disclosure(s) of any document incorporated herein by reference, the disclosure of the present application shall govern. The foregoing detailed description and examples have been given for clarity of understanding only. No unnecessary limitations are to
10 be understood therefrom. The invention is not limited to the exact details shown and described, for variations obvious to one skilled in the art will be included within the invention defined by the claims.

What is claimed is:

1. A method comprising administering a compound to a subject to reduce the availability of nickel in the subject, wherein the compound comprises dimethylglyoxime (DMG).
2. The method of claim 1, wherein the subject is suffering from or susceptible to a pathogenic infection.
3. The method of claim 2, wherein the pathogenic infection comprises infection with a bacterium that comprises a nickel-containing enzyme, a fungus that comprises a nickel-containing enzyme, or a non-fungal eukaryotic pathogen that comprises a nickel-containing enzyme.
4. The method of claim 2, wherein the pathogenic infection comprises infection with
Acinetobacter baumannii, *Enterococcus faecium*, *Escherichia coli*, *Helicobacter pylori*, *Haemophilus influenzae*, *Neisseria gonorrhoeae*, *Streptococcus pneumoniae*, a *Campylobacter* species, an *Enterobacter* species, a *Klebsiella* species, a *Morganella* species, a *Proteus* species, a *Providencia* species, a *Pseudomonas* species, a *Salmonella* species, a *Serratia* species, a *Shigella* species, or a *Staphylococcus* species, or a combination thereof;
Cryptococcus neoformans, *Cryptococcus gattii*, *Coccidioides posadasii*, *Histoplasma capsulatum*, or *Paracoccidioides brasiliensis*, or a combination thereof; or
Pythium insidiosum, *Leishmania major*, *Leishmania donovani*, or *Trypanosoma cruzi*, or a combination thereof.
5. The method of claim 2, wherein the pathogenic infection comprises infection with a multi-drug resistant pathogen.
6. The method of claim 1, wherein the subject is suffering from or susceptible to β -amyloid peptide aggregation.
7. The method of claim 6, wherein the subject is suffering from or susceptible to Alzheimer's Disease or Down Syndrome or both.

8. The method of claim 1, wherein the subject is suffering from or susceptible to a nickel allergy.
9. The method of claim 1, wherein the subject comprises nickel-resistance bacteria in their microbiome.
10. The method of claim 1, wherein the DMG comprises soluble DMG.
11. The method of claim 10, wherein the soluble DMG comprises disodium salt DMG and/or disodium salt octahydrate DMG.
12. The method of claim 1, wherein the subject comprises a human or an animal.
13. The method of claim 12, wherein the animal comprises a chicken.
14. The method of claim 1, wherein the method further comprises administering an additional active agent to the subject.
15. The method of claim 14, wherein the additional active agent comprises a metallic ion or an antibacterial compound that produces a metallic ion.
16. The method of claim 15, wherein the metallic ion comprises a divalent cation.
17. The method of claim 14, wherein the additional active agent comprises copper.
18. A method of treating or preventing a pathogenic infection in a subject, the method comprising administering dimethylglyoxime (DMG) to the subject.
19. The method of claim 18, wherein the DMG comprises soluble DMG.
20. The method of claim 19, wherein the soluble DMG comprises disodium salt DMG and/or disodium salt octahydrate DMG.

21. The method of claim 18, wherein the pathogenic infection comprises infection with a bacterium that comprises a nickel-containing enzyme, a fungus that comprises a nickel-containing enzyme, or a non-fungal eukaryotic pathogen that comprises a nickel-containing enzyme.

22. The method of claim 18, wherein the pathogenic infection comprises infection with

Acinetobacter baumannii, *Enterococcus faecium*, *Escherichia coli*, *Helicobacter pylori*, *Haemophilus influenzae*, *Neisseria gonorrhoeae*, *Streptococcus pneumoniae*, a *Campylobacter* species, an *Enterobacter* species, a *Klebsiella* species, a *Morganella* species, a *Proteus* species, a *Providencia* species, a *Pseudomonas* species, a *Salmonella* species, a *Serratia* species, a *Shigella* species, or a *Staphylococcus* species, or a combination thereof;

Cryptococcus neoformans, *Cryptococcus gattii*, *Coccidioides posadasii*, *Histoplasma capsulatum*, or *Paracoccidioides brasiliensis*, or a combination thereof; or

Pythium insidiosum, *Leishmania major*, *Leishmania donovani*, or *Trypanosoma cruzi*, or a combination thereof.

23. The method of claim 18, wherein the bacterial infection comprises infection with a multi-drug resistant pathogen.

24. The method of claim 18, wherein the subject comprises a human or an animal.

25. The method of claim 24, wherein the animal comprises a chicken.

26. The method of any claim 18, wherein the method further comprises administering an additional active agent to the subject.

27. The method of claim 26, wherein the additional active agent comprises a metallic ion or an antibacterial compound that produces a metallic ion.

28. The method of claim 27, wherein the metallic ion comprises a divalent cation.

29. The method of claim 28, wherein the additional active agent comprises copper.

30. The method of claim 18, wherein DMG is administered orally or intravenously.
31. The method of claim 30, and wherein the method comprises administering DMG orally, and wherein the DMG is presented as a capsule.
32. A method of treating or preventing β -Amyloid peptide aggregation in a subject, the method comprising administering dimethylglyoxime (DMG) to the subject.
33. The method of claim 32, wherein the DMG comprises soluble DMG.
34. The method of claim 33, wherein the soluble DMG comprises disodium salt DMG, or disodium salt octahydrate DMG, or both.
35. The method of claim 32, wherein the subject is suffering from or susceptible to Alzheimer's Disease or Down Syndrome or both.
36. The method of claim 32, wherein DMG is administered in combination with another anti-dementia therapy.
37. The method of claim 32, wherein DMG is administered orally or intravenously.
38. The method of claim 37, wherein the method comprises administering DMG orally, and wherein the DMG is presented as a capsule.
39. A method of treating or preventing nickel allergy in a subject, the method comprising administering dimethylglyoxime (DMG) to the subject.
40. The method of claim 39, wherein the DMG comprises soluble DMG.
41. A method of treating or preventing obesity in a subject, the method comprising administering dimethylglyoxime (DMG) to the subject.

42. The method of claim 41, wherein the DMG comprises soluble DMG.
43. A method of altering the balance of bacteria in the subject's microbiome, the method comprising administering dimethylglyoxime (DMG) to the subject.
44. The method of claim 43, wherein the DMG comprises soluble DMG.
45. A method of disrupting a biofilm or preventing biofilm formation, the method comprising treating a surface with dimethylglyoxime (DMG).
46. The method of claim 45, wherein the biofilm comprises a *Campylobacter* species, *Helicobacter pylori*, a *Klebsiella* species, a *Proteus* species, a *Pseudomonas* species, a *Salmonella* species, or a *Staphylococcus* species, or a combination thereof.
47. The method of claim 45, wherein the method comprises treating a surface after the formation of a biofilm, wherein treatment results in the death of bacterial cells within the biofilm.
48. A pharmaceutical composition comprising a chelator, the chelator comprising soluble DMG, wherein the pharmaceutical composition is formulated for oral or intravenous administration.
49. The pharmaceutical composition of claim 48, wherein the soluble DMG comprises disodium salt DMG, or disodium salt octahydrate DMG, or both.
50. The pharmaceutical composition of claim 48, the chelator further comprising EDTA.
51. The pharmaceutical composition of claim 48, the pharmaceutical composition further comprising an additional active agent.
52. The pharmaceutical composition of claim 51, wherein the additional active agent comprises a metallic ion or an antibacterial compound that produces a metallic ion.

53. The pharmaceutical composition of claim 52, wherein the metallic ion comprises a divalent cation.

54. The pharmaceutical composition of claim 51, wherein the additional active agent comprises copper.

FIG. 1A

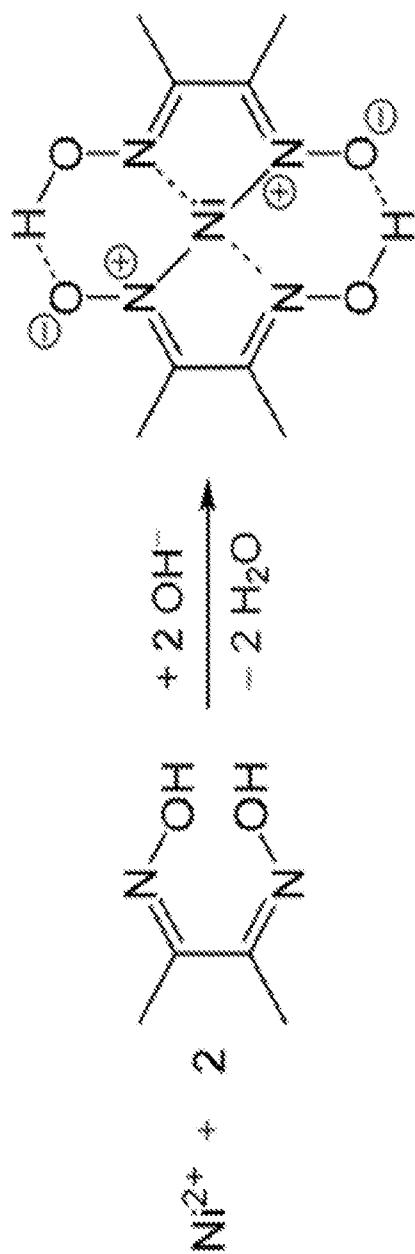


FIG. 1B

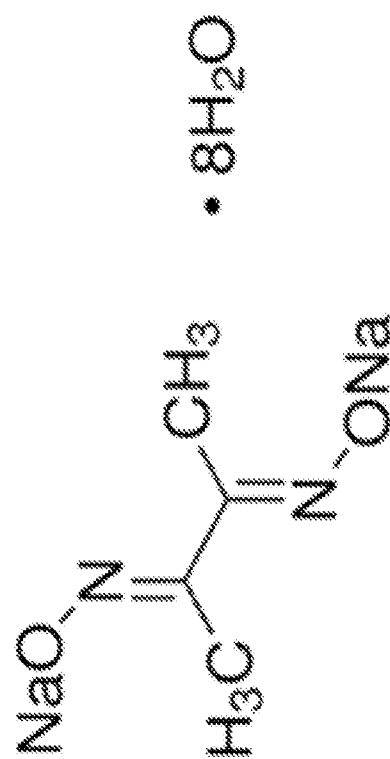


FIG. 2

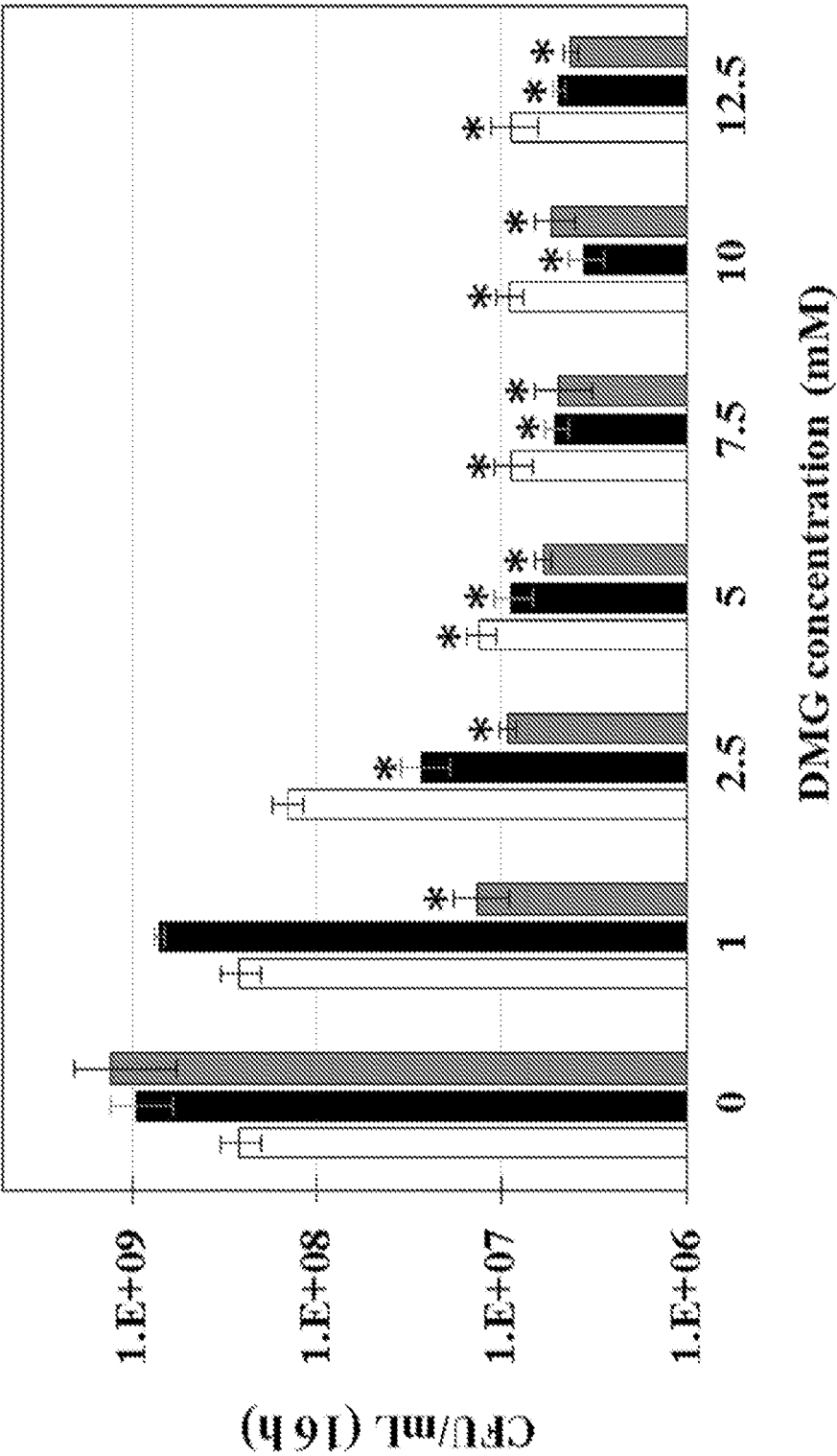


FIG. 3A

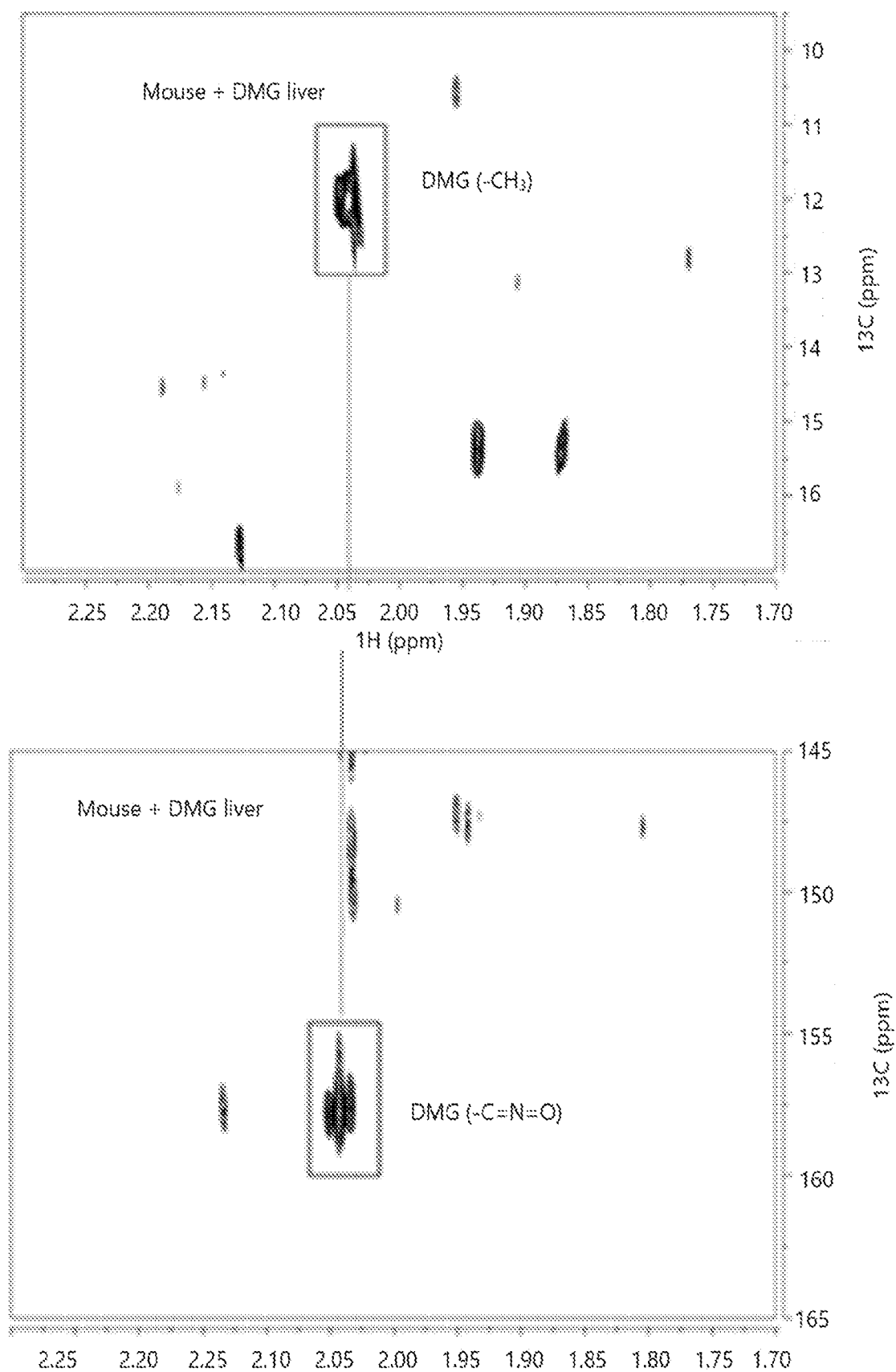


FIG. 3B

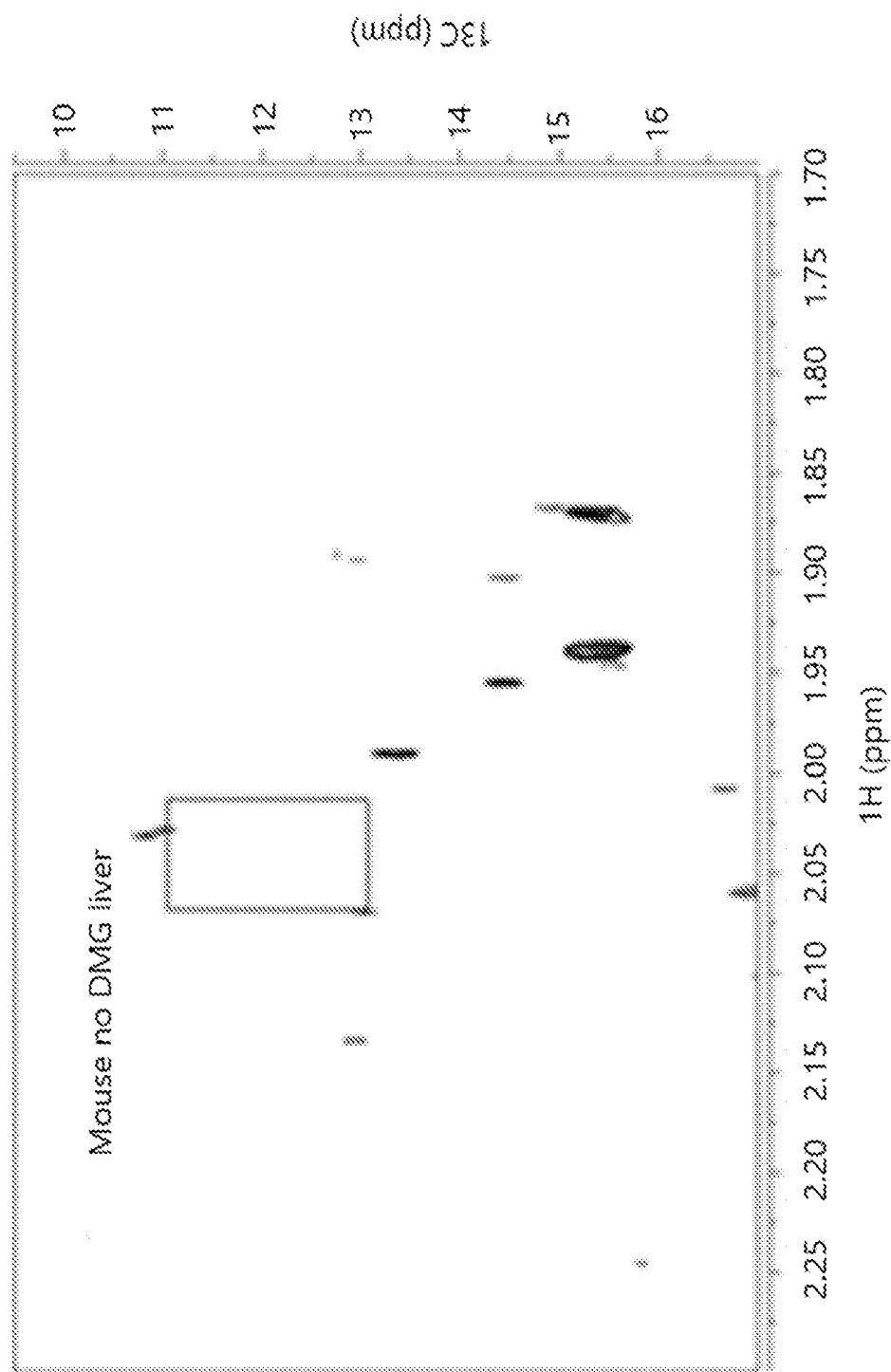


FIG. 4B

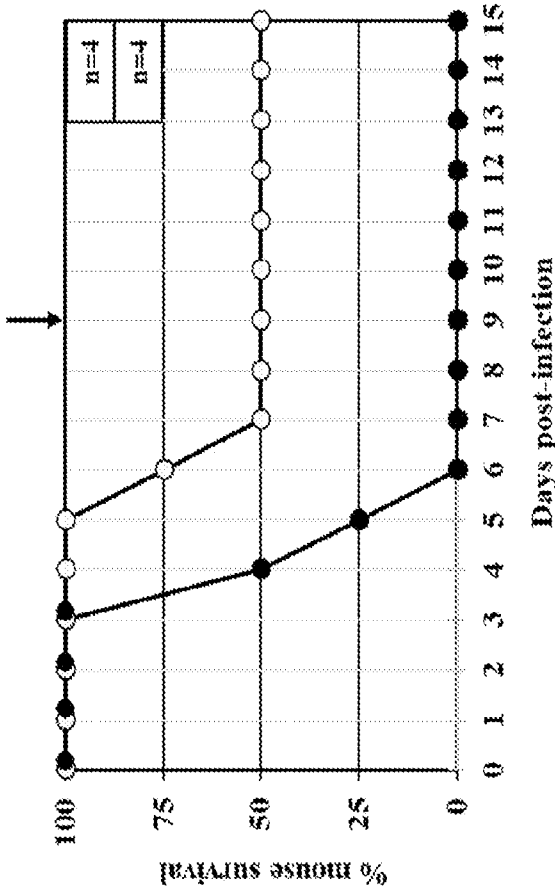
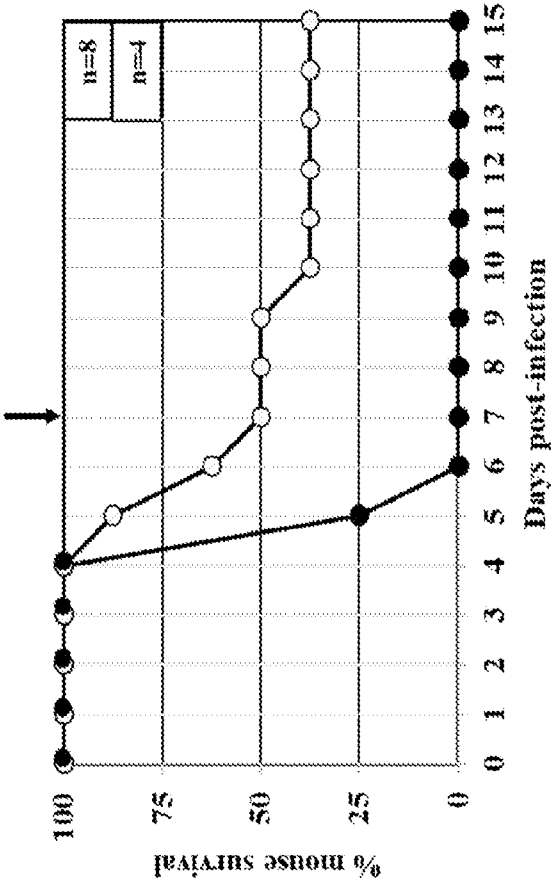


FIG. 4A



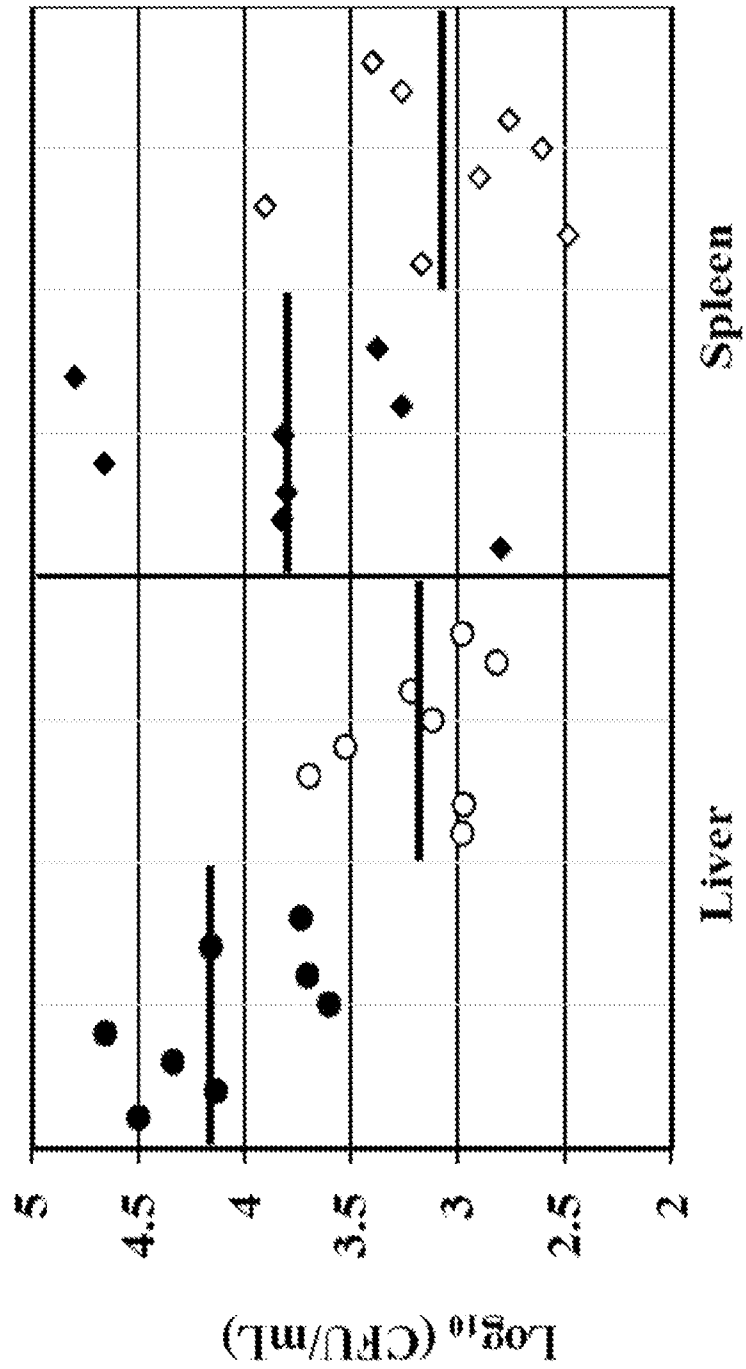


FIG. 5

FIG. 6

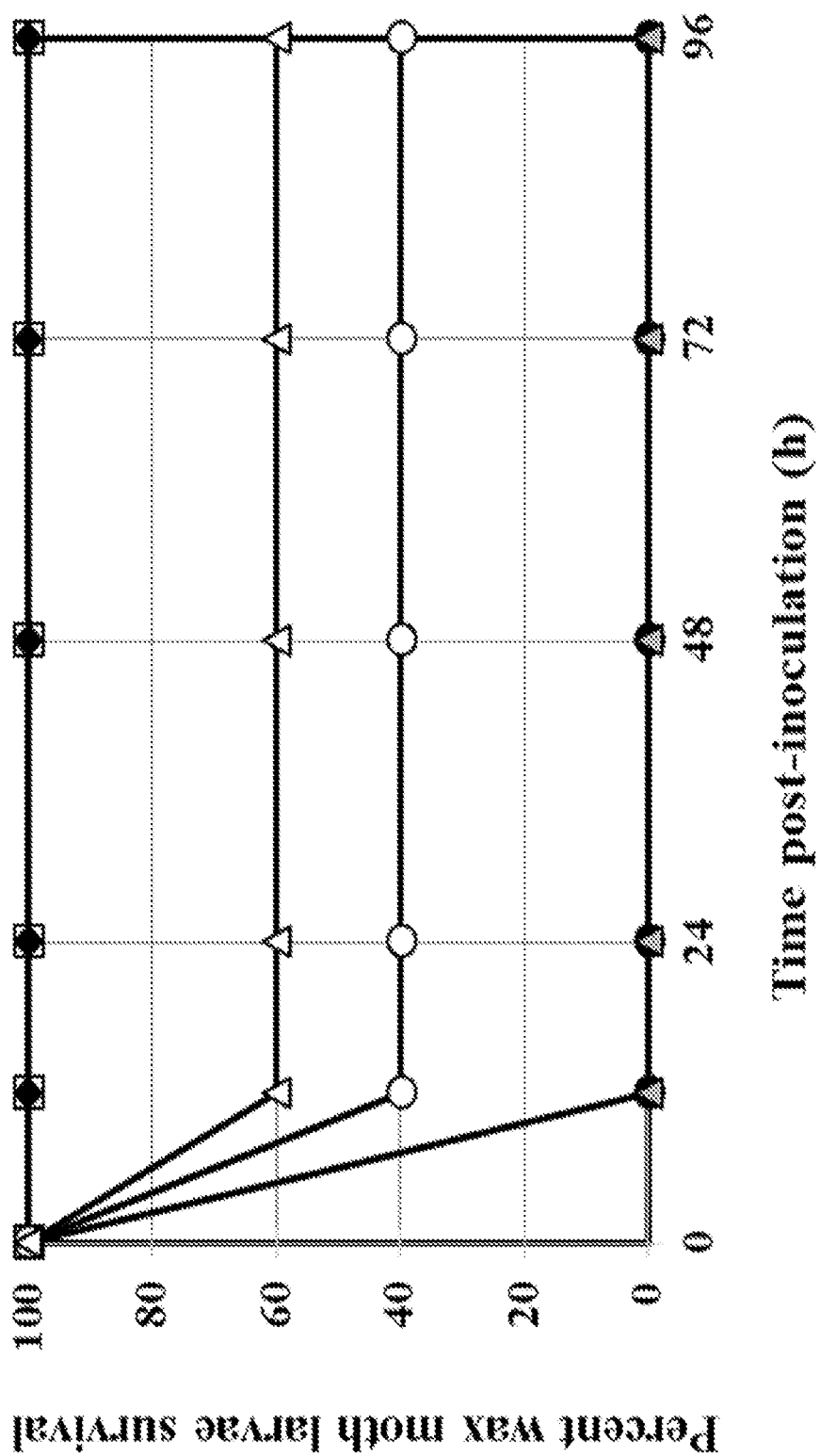


FIG. 7

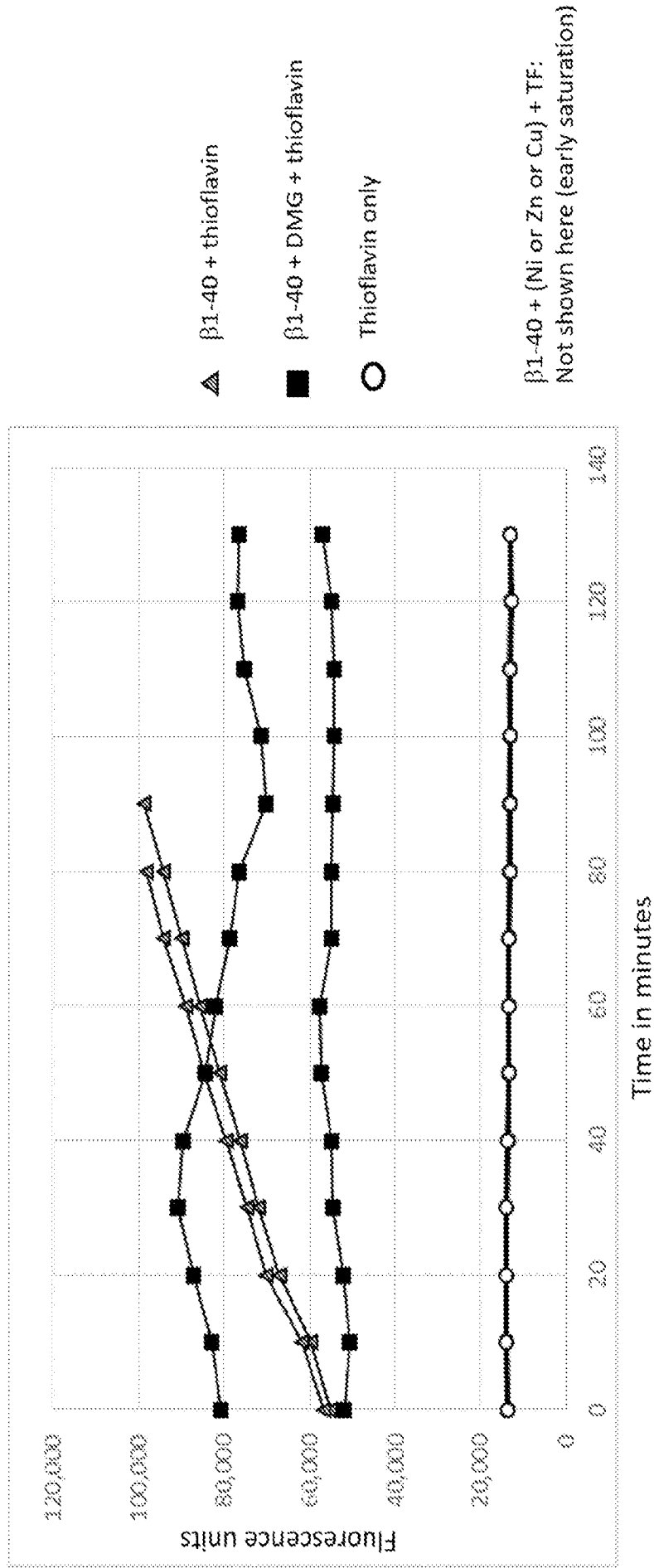


FIG. 8

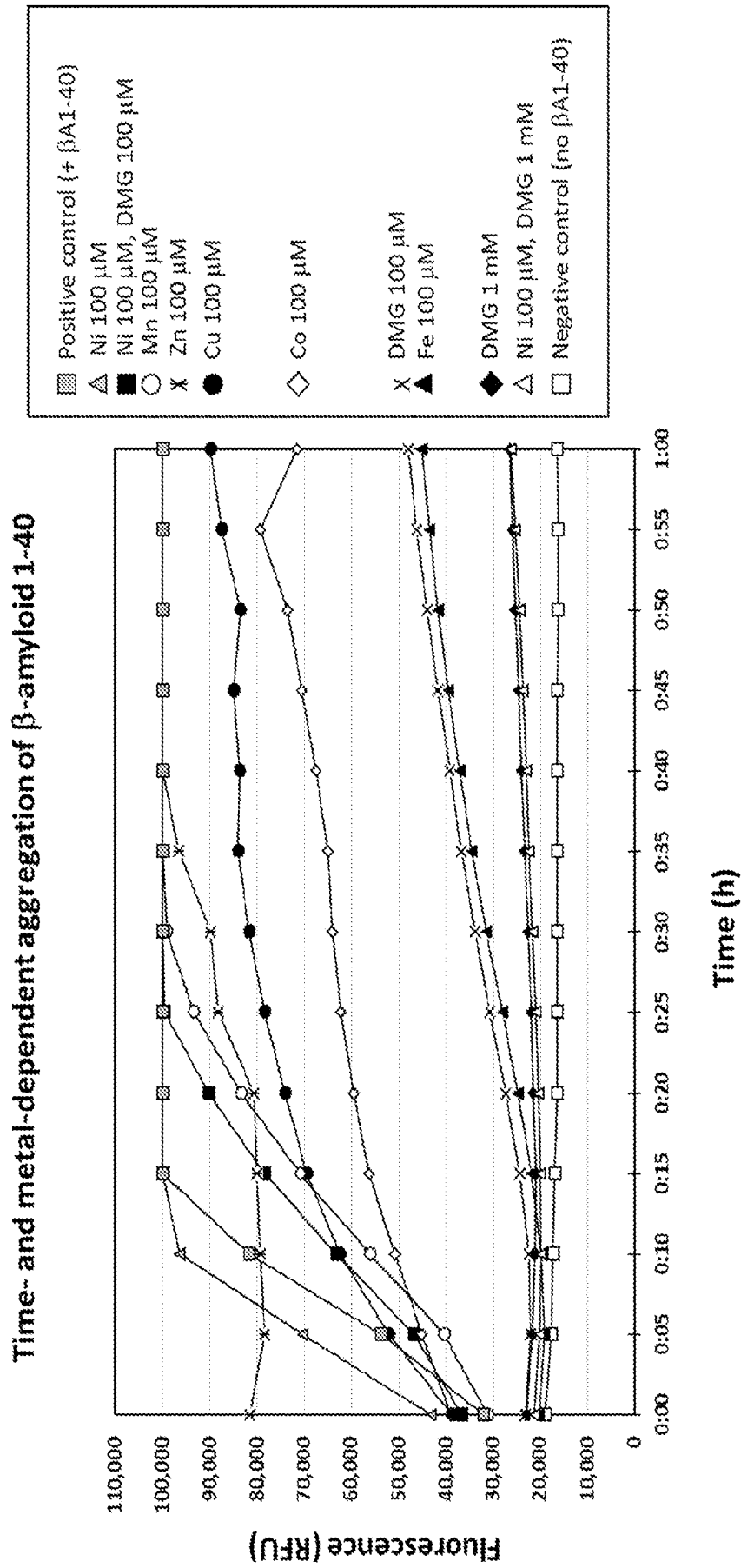


FIG. 9

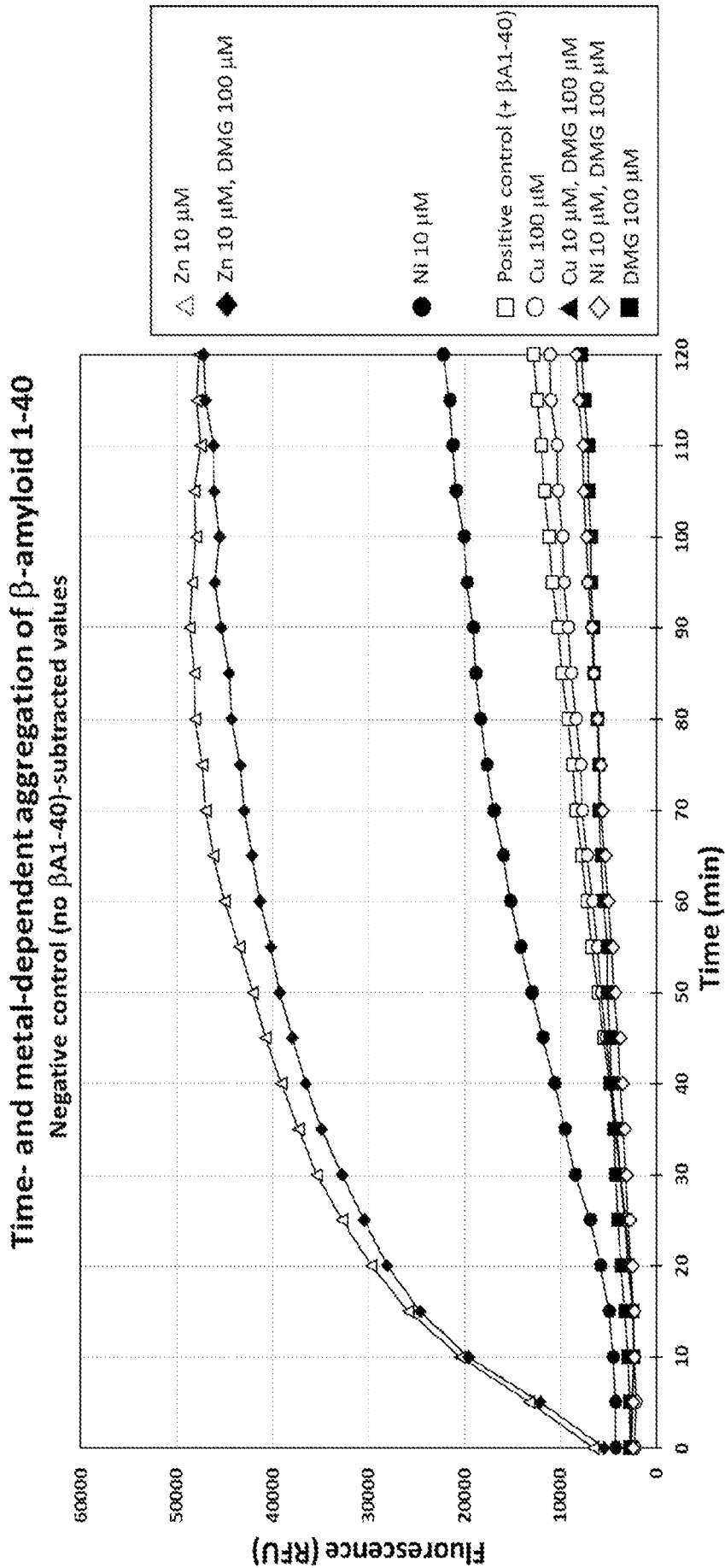


FIG. 10

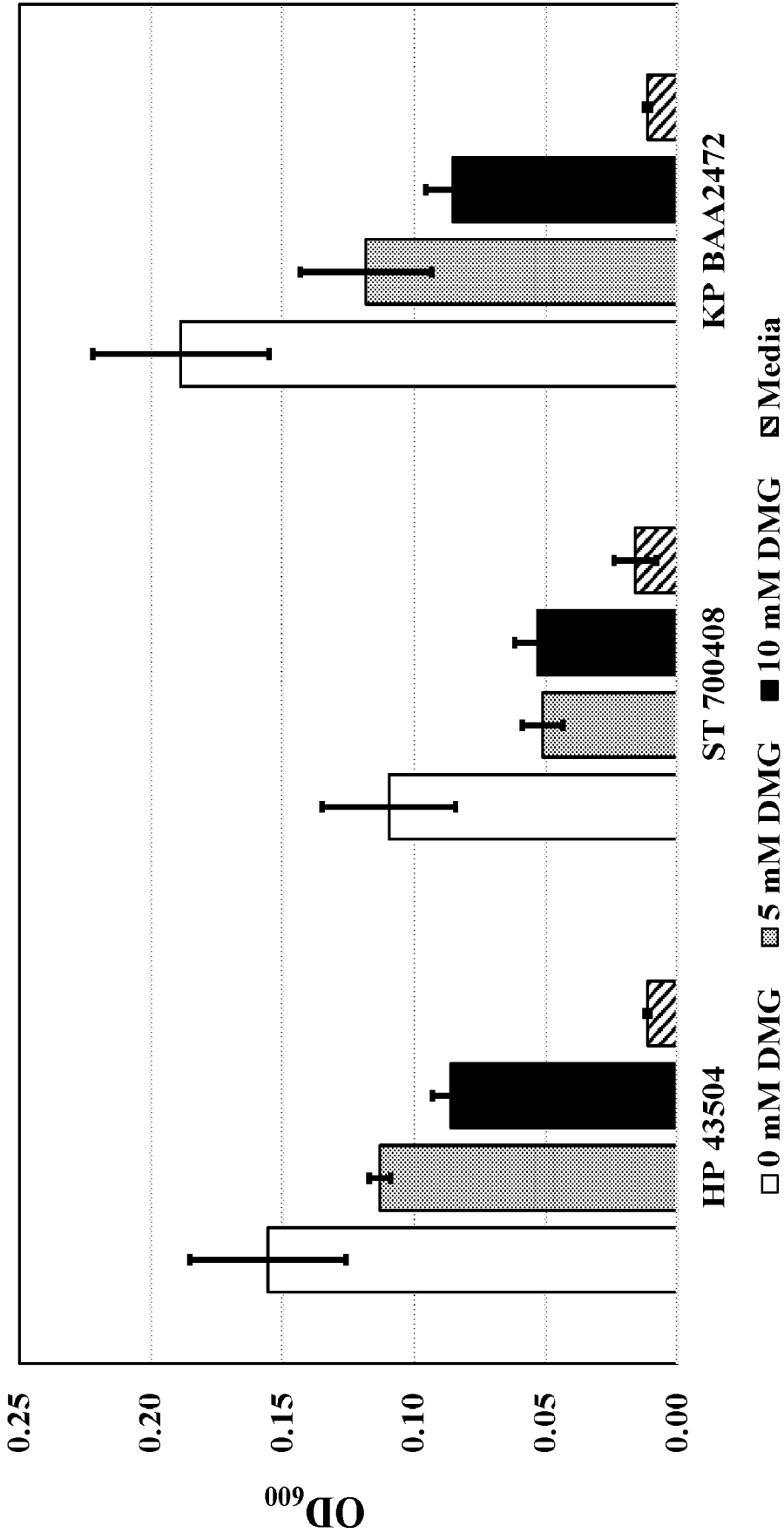


FIG. 11

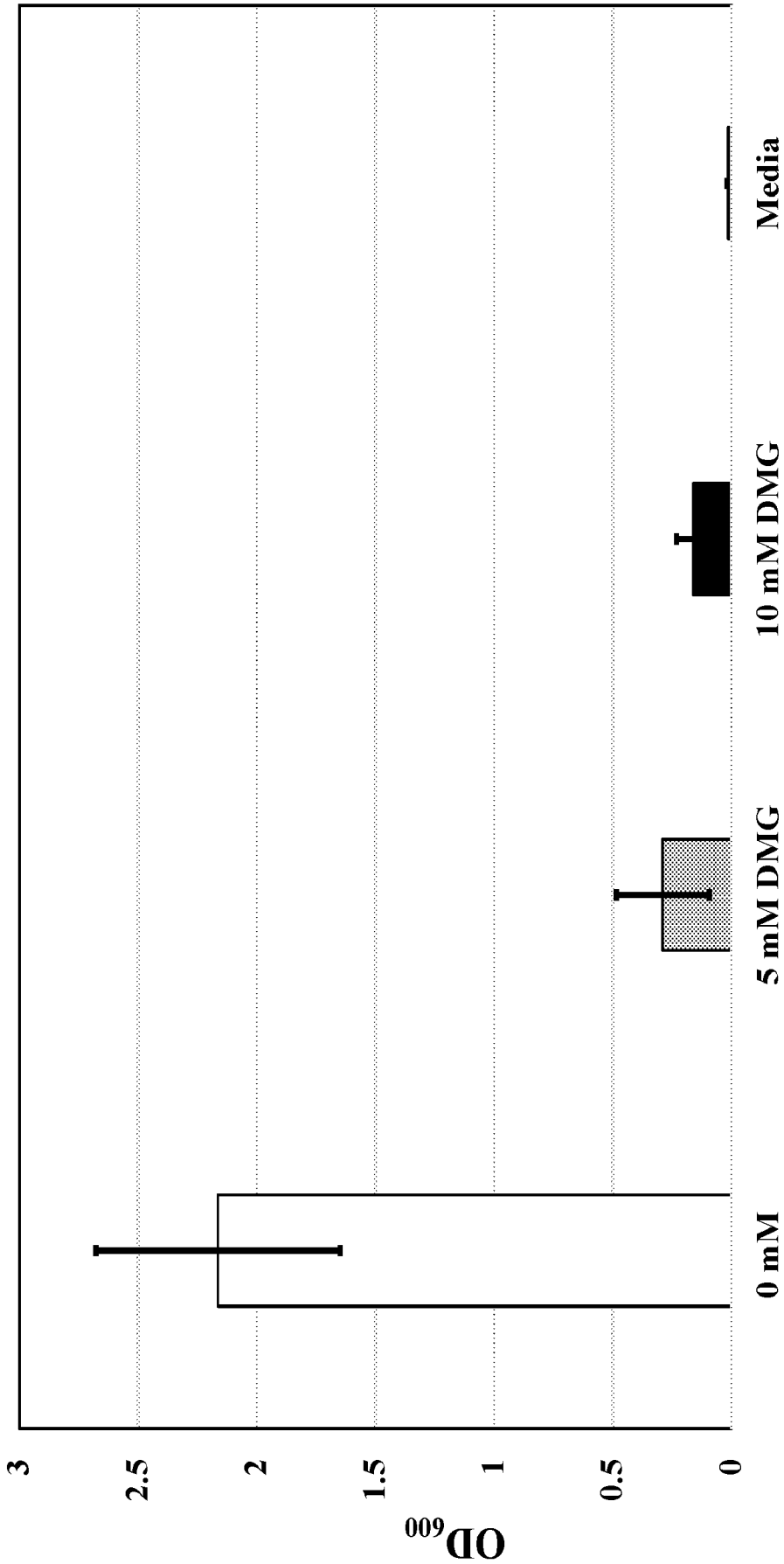


FIG. 12A

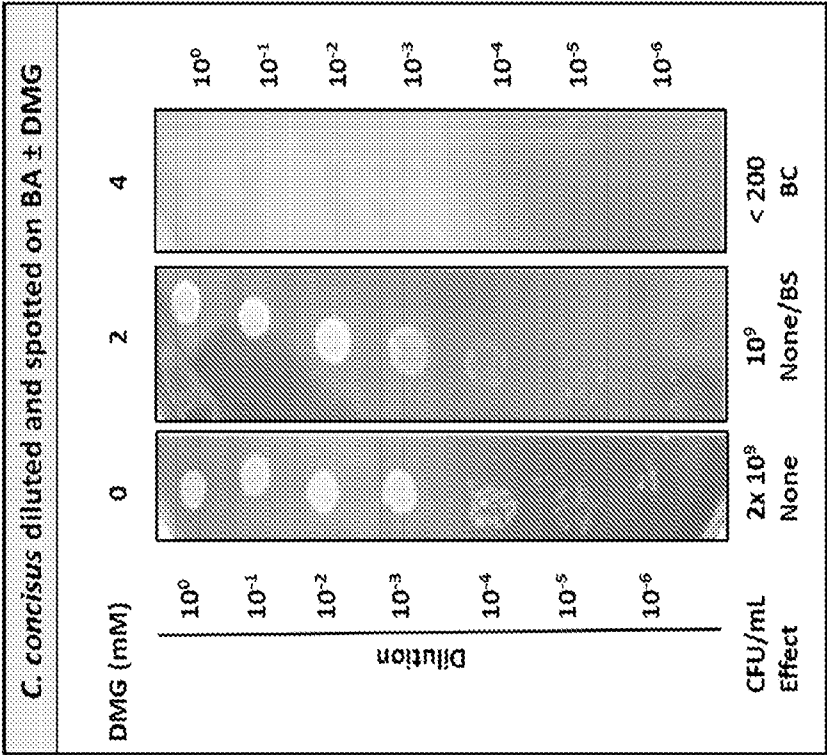
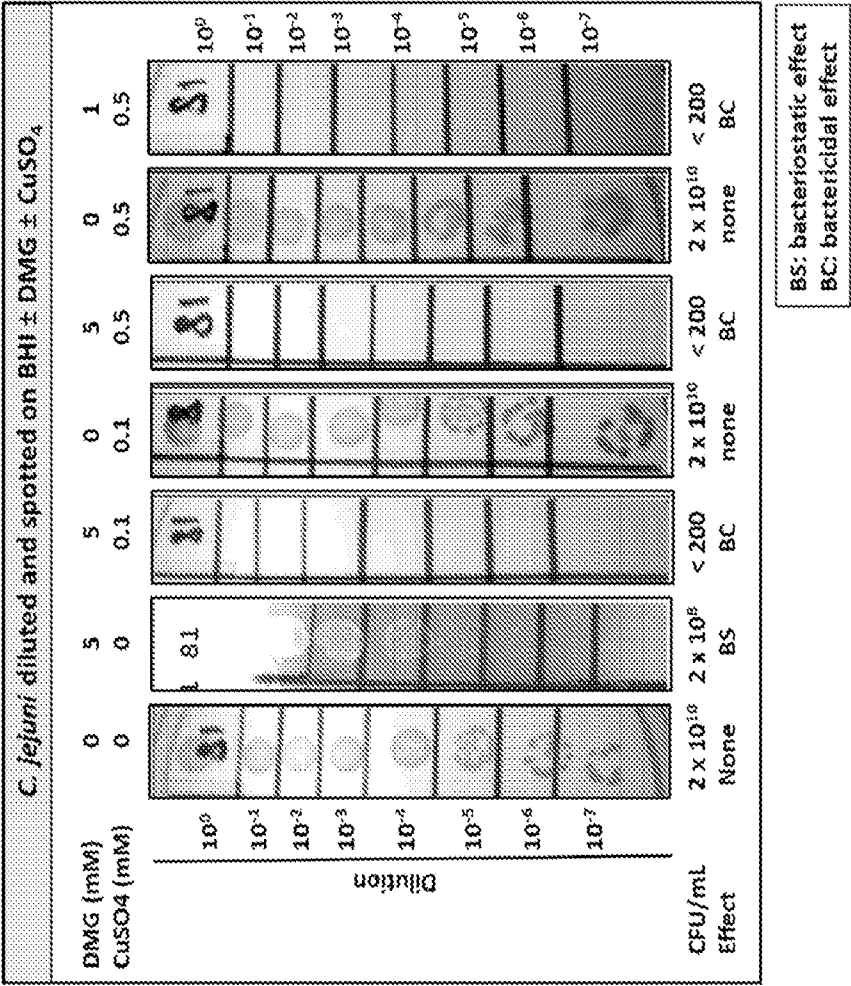


FIG. 12B



BS: bacteriostatic effect
BC: bactericidal effect

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 20/30483

A. CLASSIFICATION OF SUBJECT MATTER

IPC - A61K 31/15; A23K 20/105; A23K 20/20; A61K 33/34 (2020.01)

CPC - A61K 31/15; A23K 20/105; A23K 20/20; A61K 33/34; A61P 3/02

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)

See Search History document

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

See Search History document

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

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2003/0108927 A1 (LEISHMAN) 12 June 2003 (12.06.2003); para [0003], [0032], [0042], [0111], [0136], [0153], [0155], [0176], [0181]-[0182]; claims 1, 3-4, 7, 10, 12	1-4, 12, 14, 18, 21-22, 24, 26, 30-31
--		
Y		5, 10-11, 13, 15-17, 19-20, 23, 25, 27-29
Y	WO 2017/222335 A1 (KONKUK UNIVERSITY INDUSTRIAL COOPERATION CORPORATION) 28 December 2017 (28.12.2017); translation, pg 5, para 21; pg 10 para 7	5, 23
Y	WO 2013/114089 A1 (C-TECH INNOVATION LIMITED) 08 August 2013 (08.08.2013); pg 9 in 19-21	10-11, 19-20
Y	US 2015/0157594 A1 (DR. BATA ZRT.) 11 June 2015 (11.06.2015); para [0001], [0019], [0030], [0061]	13, 15-17, 25, 27-29
A, P	BENOIT S. L. et al., "Nickel chelation therapy as an approach to combat multi-drug resistant enteric pathogens", Scientific Reports, 25 September 2019, volume 9, article 13851, 10 pp.; retrieved from the Internet: < DOI: 10.1038/s41598-019-50027-0 >, see entire document	1-5, 10-31
A	HEO J. et al., "Evidence for a Ligand CO That Is Required for Catalytic Activity of CO Dehydrogenase from Rhodospirillum rubrum", Biochemistry, 2000, volume 39, issue 27, pp. 7956-7963; retrieved from the Internet: < DOI: 10.1021/bi992958g > see entire document	1-5, 10-31

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

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

"D" document cited by the applicant in the international application

"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

18 August 2020

Date of mailing of the international search report

14 SEP 2020

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents

P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

Authorized officer

Lee Young

Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 20/30483

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

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Please see attached sheet--

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-5 and 10-31

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

Attachment to Box.No.III:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I+: Claims 1-44, directed to a method comprising administering a compound to a subject to reduce the availability of nickel in the subject, wherein the compound comprises dimethylglyoxime (DMG), wherein the subject is susceptible to a pathogenic infection, Alzheimer's disease or suffering from a nickel allergy or comprises nickel-resistance bacteria in their microbiome; a method of treating or preventing a pathogenic infection, Beta-Amyloid peptide aggregation, nickel allergy, obesity in a subject and to a method of altering the balance of bacteria in the subject's microbiome the method comprising administering dimethylglyoxime (DMG) to the subject.

The method will be searched to the extent that the method encompasses preventing or treating a pathogenic infection in a subject. It is believed that claims 1-5 and 10-31 read on this first named invention, and thus these claims will be searched without fee to the extent that they encompass preventing or treating a pathogenic infection. Applicant is invited to elect additional method(s), wherein each additional method elected, will require one additional invention fee. Applicants must specify the claims that encompass any additionally elected method. Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the '+' group(s) will result in only the first claimed invention to be searched. Additionally, an exemplary election wherein different actual variables are selected is suggested. An exemplary election would be a method for treating or preventing P-Amyloid peptide aggregation in a subject, the method comprising administering dimethylglyoxime (DMG) to the subject (i.e., claims 1, 6-7, 10-17 and 32-38).

Group II: Claims 45-47, directed to a method of disrupting a biofilm or preventing biofilm formation, the method comprising treating a surface with dimethylglyoxime (DMG).

Group III: Claims 48-54, directed to a pharmaceutical composition comprising a chelator, the chelator comprising soluble DMG, wherein the pharmaceutical composition is formulated for oral or intravenous administration.

The group of inventions listed above do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features:

Group I+ includes the technical feature of a unique method of treating a subject, which is not required by any other invention of Group I+, or by Groups II and III.

Group II includes the technical feature of disrupting or preventing biofilm on a surface, not required by Groups I+ and III.

Group III includes the technical feature of a pharmaceutical composition comprising soluble DMG, not required by Group II.

Common technical features:

Groups I+-III share the technical feature of the nickel chelator, dimethylglyoxime.

This shared technical feature, however, do not provide a contribution over the prior art, as being anticipated by US 2003/0108927 A1 to Leishman (hereinafter Leishman), which discloses the nickel chelator, dimethylglyoxime (claims 3, 4).

The inventions of Groups I+ share the technical feature of a method comprising administering a compound to a subject to reduce the availability of nickel in the subject, wherein the compound comprises dimethylglyoxime (DMG), wherein the subject is susceptible to a pathogenic infection, Alzheimer's disease or suffering from a nickel allergy or comprises nickel-resistance bacteria in their microbiome; a method of treating or preventing a pathogenic infection, Beta-Amyloid peptide aggregation, nickel allergy, obesity in a subject or a method of altering the balance of bacteria in the subject's microbiome the method comprising administering dimethylglyoxime (DMG) to the subject. This shared technical feature, however, do not provide a contribution over the prior art, as being obvious over Leishman, which discloses a method comprising administering a compound to a subject susceptible to a pathogenic infection, to reduce the availability of nickel in the subject, wherein the compound comprises dimethylglyoxime (DMG) (claim 1: "method for the prevention of an infectious disease in a human subject, comprising the steps of (a) providing a pharmaceutical composition comprising at least one substance selected from the group consisting of ... CODH chelator; and (b) administering the composition of (a) to the patient in a form that allows uptake by cells of the subject"; claim 4: "the CODH chelator is selected from the group consisting of ... dimethylglyoxime"; para [0153]: "the functions of the clusters in CODH ... the Nickel (Ni) ion in one of the clusters (subsequently named the A-cluster) could be selectively removed by adding a metal chelating agent"; also para [0155], Table 1: "Ni-specific chelator, dimethylglyoxime. Ni in the CO(L)-deficient CODH can be removed by treatment"). Whereas Leishman does not specifically disclose a single example or specific embodiment of the said method, in view of the above disclosure, it would have been obvious for the person of ordinary skill in the art to design the said method protocol, in the course of routine experimentation, in order to effectively prevent an infectious disease in a subject (claim 1).

As said nickel chelator and method were known in the art at the time of the invention, these cannot be considered special technical features, that would otherwise unify the inventions of Group I+-III.

The inventions of Group I+-III, thus lack unity under PCT Rule 13.