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Caehnorhabditis Elegans: A Low-Cost In Vivo Animal Model for Efficacy Studies of Novel Antibiotics

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CAEHNORHABDITIS ELEGANS: A LOW-COST *IN VIVO* ANIMAL MODEL FOR EFFICACY STUDIES OF NOVEL ANTIBIOTICS

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INTRODUCTION

Antibiotic Resistance

Since the 1940s, antibiotics have greatly reduced the adverse effects of infectious diseases caused by microbes[1]. However, due to excessive, and often incorrect, use of known antibiotics, many organisms have adapted antibiotic resistance. Currently, over 70% of known infectious bacteria are resistant to at least one antibiotic [16]. In the U.S., 90,000 deaths occur each year due to infection by bacteria resistant to antibiotics. This number has increased by nearly 75,000 in the last 20 years. It is necessary, therefore, to continue developing new antibiotics in an effort to keep up with increasing antibiotic resistance. Traditional *in vitro* and whole cell drug screens are inefficient[13]. Unlike *in vitro* studies, *in vivo* studies can rule out toxic compounds early in analysis. They can also test compounds in the presence of important host/pathogen relationships. The action of an antimicrobial can therefore be viewed within the context of the whole organism, and not just at the drug-receptor site, as *in vitro*[15]. Current *in vivo* testing is done in small mammals, such as mice. Mice, however, are resource intensive to maintain. Often, the antibiotic compounds being tested are toxic to the mice, killing them and creating a need for new mice[8]. Our lab group has proposed the use of the nematode *Caenorhabditis elegans* for *in vivo* tests as an intermediate step prior to testing in mammals.

Caenorhabditis elegans

Caenorhabditis elegans is a 1 mm long, translucent nematode. *C. elegans* have been used extensively as a model organism for studies in genetics for about 40 years[3]. Therefore, its entire genome has been sequenced, and much is known about its general

use in a laboratory setting. Its small size and rapid life cycle make it easy to maintain and less costly than mice as an organism for *in vivo* testing. *C. elegans* also has appeal as model organisms because it is free of the ethical concerns. Its small size allows for large-scale experiments to be done using a small apparatus such as agar on microtitre plates or in liquid medium in 96-well plates[15]. It grows at room temperature in regular air on a diet of OP50 strain of *E. coli*, but its natural diet is unknown[8]. It is proposed that it may be necromenic, feasting on microorganisms associated with rotting organic matter. On a regular diet, the worms generally have a lifecycle of about 15 days, allowing for preparation of useable colonies in a short amount of time. They generally require approximately three days to mature from egg to adult.

C. elegans has a simple and straightforward anatomy, but still maintains many different tissues and organs, such as muscle, intestine, glands, hypodermis, nervous system, and reproductive system[10,11]. There is strong conservation between *C. elegans* and mammals in cellular and molecular pathways[15]. Its transparency also allows for easy viewing of the inner organs of the worm, as well as easy use of fluorescent dyes and markers. We propose, therefore, the use of *C. elegans* as a model organism for *in vivo* efficacy testing of novel antibacterial compounds.

Enterococcus faecalis

Enterococcus faecalis is a gram positive cocci shaped bacteria[5]. It is one of the three leading causes of nosocomial infection. It is also one of the most antibiotic resistant bacteria known. Strains exist that are resistant to vancomycin, termed Vancomycin Resistant Enterococcus (VRE). We have previously synthesized and tested a number of compounds, collectively termed cationic naphthoquinone analogs,

that have proven effective against *E. faecalis in vitro*. This pathogenic bacteria, therefore, was selected for use in the proposed *C. elegans in vivo* model. It has been shown, in previous studies, to cause a persistent infection, taking up residence in the *C. elegans* digestive system[12].

Naphthoquinone derivatives as antibiotics

Naphthoquinone is a naturally occurring molecule with a wide range of biological activities, including uses as antibacterial, antifungal, antimalarial[14], antitumor, or as inhibitors against vitamin K dependent carboxylase, protein kinase, coenzyme Q, and as growth stimulator for bifidobacteria[19]. It is found in a number of plants used for medicinal purposes all over the world[7,9]. Naphthoquinones have been shown to act as alternative electron acceptors in the electron transport chain, causing mitochondrial dysfunction and free radical generation[19]. This dysfunction and the produced free radicals are likely instrumental in naphthoquinone cytotoxicity.

Naphthoquinone derivatives, specifically those with an attached triazole ring at the 1,4 positions, called 1-alkyl-1*H*-naphtho[2,3-*d*]triazole-4,9-diones, show promise as antibacterial agents against gram positive bacteria[12]. It is hypothesized that these compounds function as antibacterials by disrupting the bacterial membrane. Neutral analogs, however, are insoluble in aqueous media, and are therefore not very useful as biological agents. It was found, however, that by converting the compounds into cationic salts, solubility increased[6]. Their biological activity was also increased by the addition of long chain carbohydrates that increased the lipophilicity of the molecule, likely due to greater ease in disrupting bacterial plasma membranes[19].

METHODS

Antibiotics were tested *in vitro* for their Minimum Inhibitory Concentrations (MIC). This was accomplished in a 96-well microtitre plate. A two-fold series dilution of antibiotics was performed, and an equal amount of bacteria was pipetted into each well. The plate was then incubated at 35°C for 18 hours.

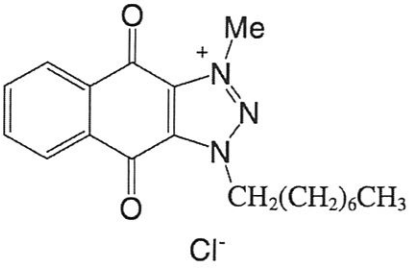
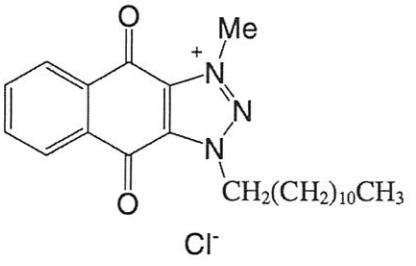
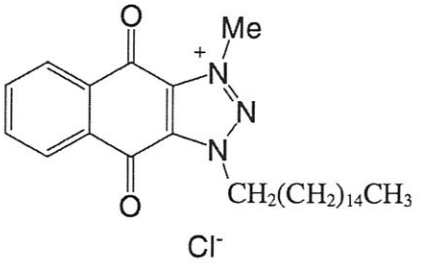
C. elegans were grown on Nematode Growth Agar (NGA) on a diet of OP50 *E. coli.* Colonies were allowed a two day growth period before infection to ensure a higher number of adult worms in the population. Infection was carried out by suspending the *C. elegans* in a solution of M9 buffer, washed of OP50 *E. coli* by centrifugation, and transferred to a plate of *E. faecalis.* Antibiotics were then administered two days after infection by *E. faecalis.* Colonies were treated separately with either neomycin, or NQM108. Antibiotic treatment was carried out by pipetting 50 µL of 2 µg/mL neomycin and 64 µg/mL NQM108. Colonies were then monitored for one week for improved health.

To determine if the antibiotics employed to heal *C. elegans* showed any toxicity against *C. elegans,* healthy *C. elegans* were administered antibiotics at 2x and 4x their MIC two days following normal growth on NGA. Colonies were monitored for one week.

Samples were viewed and documented daily using fluorescence microscopy and SYTOX fluorescent dye. SYTOX is impermeable to intact membranes, but will easily penetrate a compromised membrane[17]. Selective data is shown below corresponding to the most significant events during the week-long assay.

RESULTS AND DISCUSSION

In vitro tests

Minimum Inhibitory Concentration (MIC) in μg/mL[18]					
Antibiotic	Structure	*E. faecalis*	*VRE*	*S. aureus*	*E. coli*
Neomycin	N/A	16-32	125-250	1	4
NQM 108		125-250	125-250	8	8
NQM 103		4-8	4-8	2-4	2-4
NQM 113		2-4	4-8	0.032	0.5-1
HTB[a]	N/A	2-4	4-8	0.5-1	1

[a] HTB = hexadecyltrimethylammonium bromide

Antibacterial activity in general appears to increase with increasing alkyl chain length on the N-3 of the triazole ring. Both compounds NQM 103 and 105 show high

potency (low MIC) against *E. faecalis*, comparable to that of the control HTB. NQM 108, however, required a higher concentration, 125-250 μg/mL, to inhibit *E. faecalis* growth, but was potent against the gram negative bacteria *E. coli.* This suggests that NQM 108 functions by a different mode of action than NQM 103 or NQM 105 against *E. faecalis*. Because NQM 108 is potent against the gram positive bacteria, *S. aureus,* but not against the similarly gram positive *E. faecalis,* it is possible that its mode of action does not involve the similar bacterial membranes of the two bacteria.

In vivo tests

Worms that have been infected with *E. faecalis* were expected to fluoresce more that brightly than their healthy counterparts, due to a weakening of the *C. elegans* cuticle in an infirm state and subsequent influx of SYTOX dye. *C. elegans* infected with *E. faecalis* fluoresced as predicted (see figure 2). This can be compared to the minimal fluorescence seen in the healthy control in figure 1. Once the antibiotics, neomycin and NQM 108, were added to the NGA plates, fluorescence reduced significantly to levels comparable to the healthy control (see figures 3 and 4). This suggests that the *E. faecalis* infection has effectively been cleared. We can conclude, therefore, that membrane permeability decreased with administration of antibiotics. This is likely due to action of the antibiotics against *E. faecalis.* It can also be assumed that one effect of the *E. faecalis* infection is the weakening of the *C. elegans* cuticle, and that the cuticle regains its strength when the infection is cleared. This assumption is supported by the action of the SYTOX fluorescent dye, which did not penetrate the *C. elegans* cuticle in the healthy control or either colony that was administered antibiotics.

In the presence of antibiotics only, *C. elegans* fluorescence remained minimal (see figures 5 and 7). At 2x MIC of both neomycin and NQM 108, *C. elegans* fluorescence still remained minimal (see figures 6 and 8). This is promising, as the MIC of NQM 108 is already high (125 μg/mL). In order for these results to be fully conclusive, however, the experiment will need to be repeated with increasing multiples of the MIC until toxicity is eventually reached. The results do indicate, however, that the antibiotics are not negatively affecting *C. elegans* health in the assays involving bacteria. We can assume, therefore, that adverse health effects, specifically weakening of the *C. elegans* cuticle, arise from the bacterial infection.

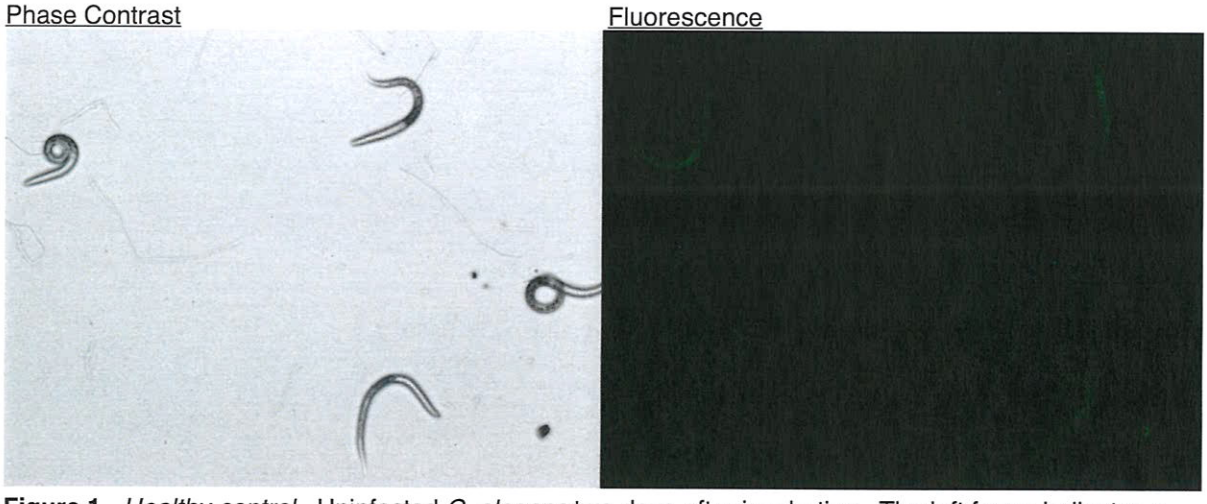


Figure 1. *Healthy control.* Uninfected *C. elegans* two days after incubation. The left frame indicates a regular phase contrast image of the *C. elegans*, while the right frame shows the same field of view, but as a fluorescent image. None of the worms in the right frame show significant fluorescence.

Phase Contrast

Fluorescence

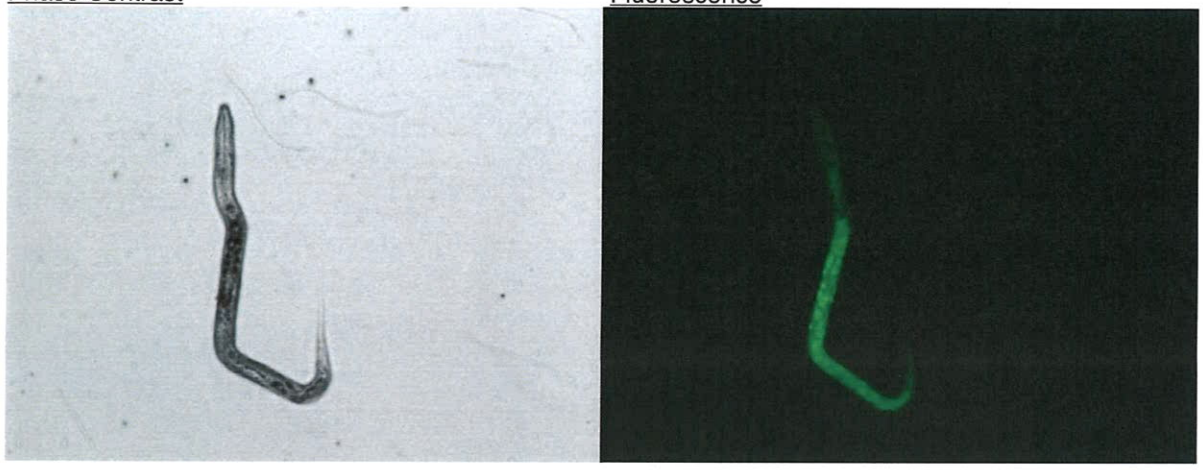


Figure 2. *Infected control. C. elegans* two days after infection with *E. faecalis*. The left frame indicates a regular phase contrast image of the *C. elegans*, while the right frame shows the same field of view, but as a fluorescent image. The right frame shows a brightly fluorescing worm, indicating a weakened outer membrane. The sharp body angles of the worm, as opposed to the smooth curves seen in Figure 1, also indicate an infirm state[12].

Phase Contrast

Fluorescence

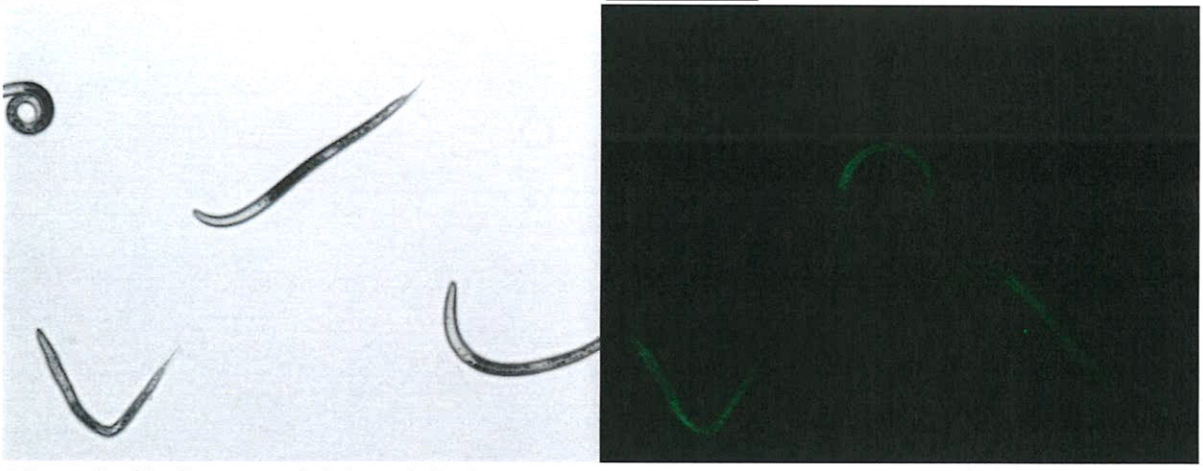


Figure 3. *Healing control.* Infected *C. elegans* six days after administration of 16 μg/mL Neomycin. This indicates the first day showing markedly decreased fluorescence. The left frame indicates a regular phase contrast image of the *C. elegans*, while the right frame shows the same field of view, but as a fluorescent image. Worms show significantly reduced fluorescence, but do appear slightly brighter than the healthy control (shown in Figure 1).

Phase Contrast

Fluorescence

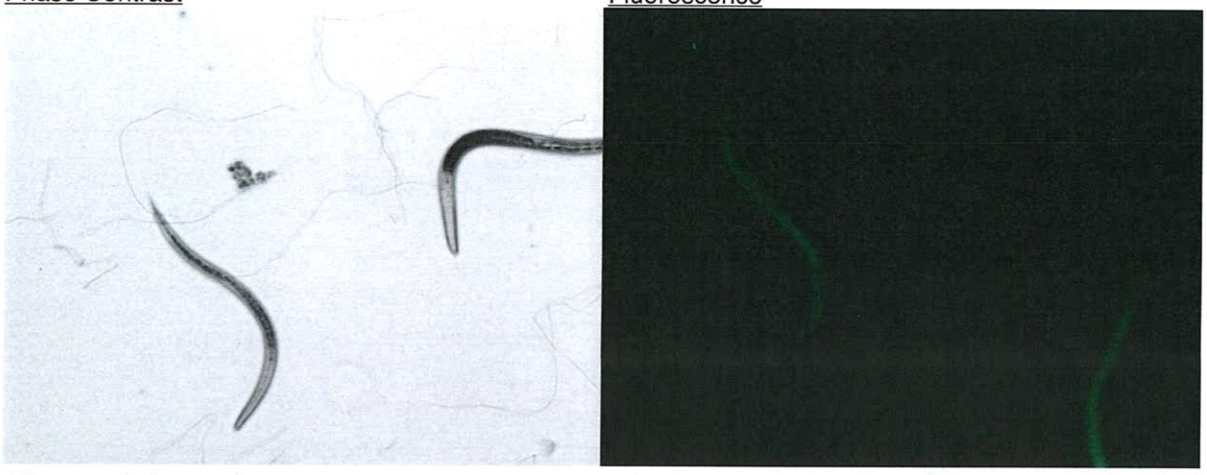


Figure 4. Infected *C. elegans* two days after administration of 125 μg/mL novel antibiotic, NQM108. This was the firstThe left frame indicates a regular phase contrast image of the *C. elegans*, while the right frame shows the same field of view, but as a fluorescent image. Worms show significantly reduced fluorescence, but do appear slightly brighter than the healthy control (shown in Figure 1). Fluorescence is comparable to Figure 3, where worms were given Neomycin, a commercially available antibiotic.

Phase Contrast

Fluorescence

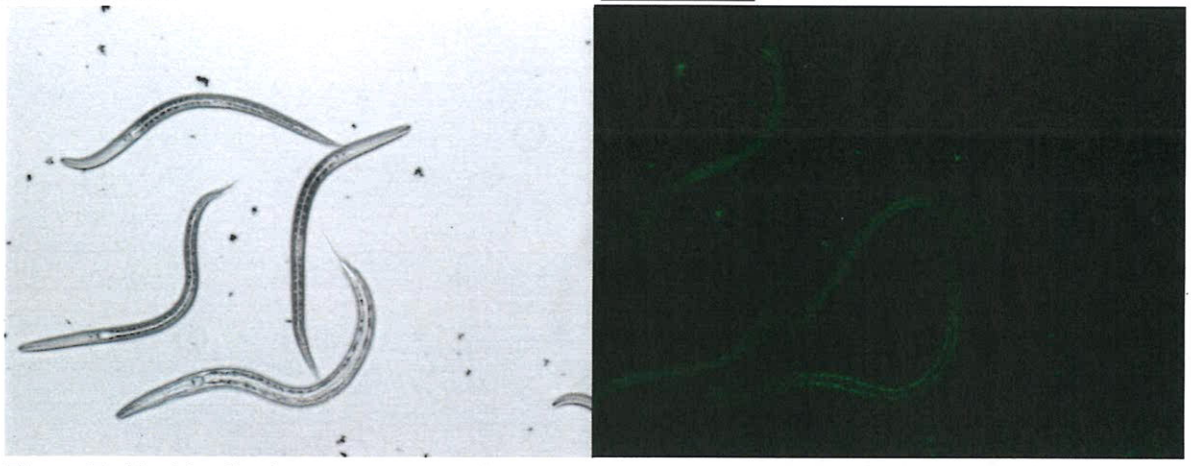


Figure 5. Healthy *C. elegans* one week after administration of 16 μg/mL of Neomycin (MIC). The left frame indicates a regular phase contrast image of the *C. elegans*, while the right frame shows the same field of view, but as a fluorescent image. None of the worms in the right frame show significant fluorescence.

Phase Contrast

Fluorescence

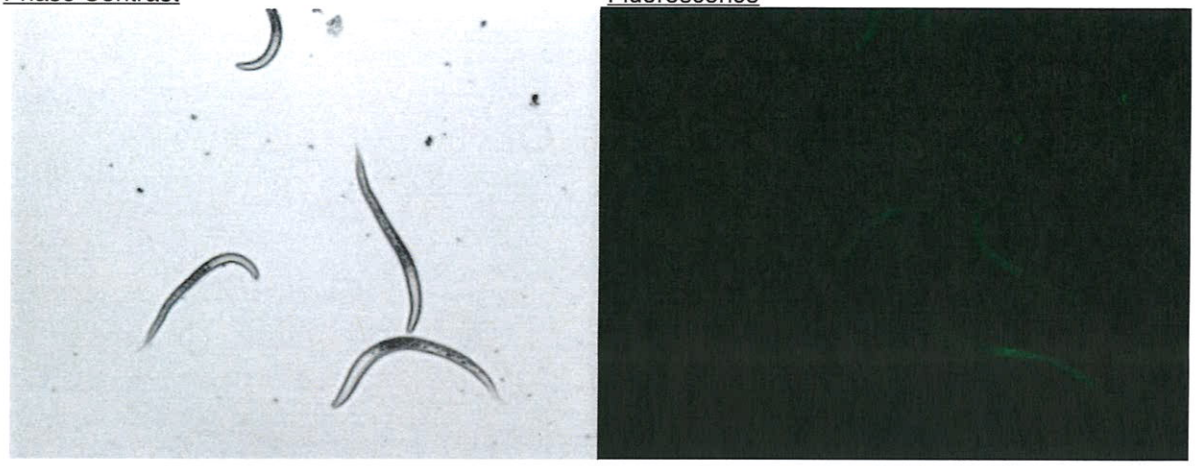


Figure 6. Healthy *C. elegans* one week after administration of 32 μg/mL of Neomycin (2x MIC). The left frame indicates a regular phase contrast image of the *C. elegans*, while the right frame shows the same field of view, but as a fluorescent image. None of the worms in the right frame show significant fluorescence.

Phase Contrast

Fluorescence

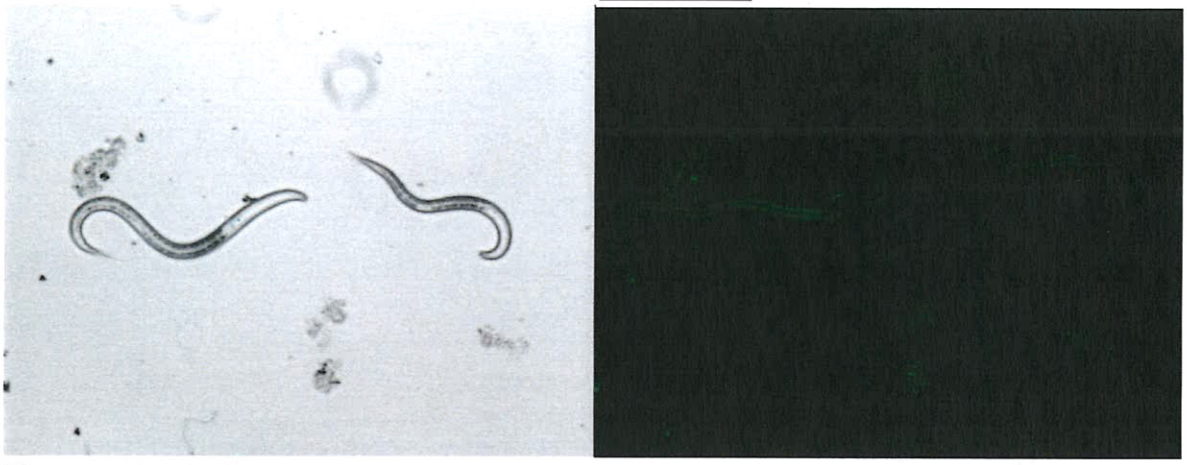


Figure 7. Healthy *C. elegans* one week after administration of 125 μg/mL of NQM 108 (MIC). The left frame indicates a regular phase contrast image of the *C. elegans*, while the right frame shows the same field of view, but as a fluorescent image. None of the worms in the right frame show significant fluorescence.

Phase Contrast

Fluorescence

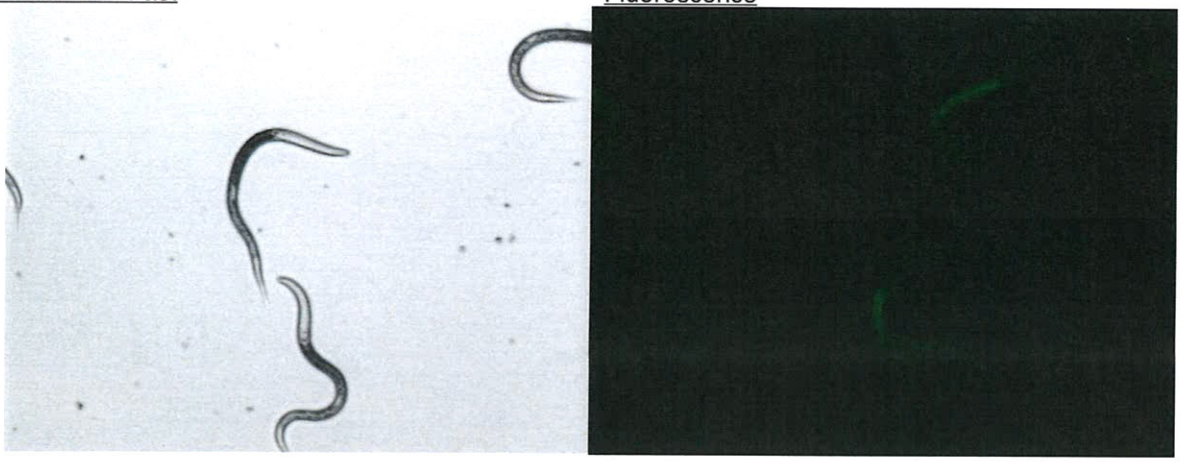


Figure 8. Healthy *C. elegans* one week after administration of 250 μg/mL of NQM 108 (2x MIC). The left frame indicates a regular phase contrast image of the *C. elegans*, while the right frame shows the same field of view, but as a fluorescent image. None of the worms in the right frame show significant fluorescence.

CONCLUSIONS

Novel cationic naphthoquinone analogs show marked antibacterial activity *in vitro,* and specifically NQM 108 showed activity *in vivo* as well. *C. elegans,* as far as they were tested, also proved effective as a model organism for *in vivo* studies, as they were shown to contract an infection, and return to a normal state following antibiotic treatment. While these results are preliminary, no major problems arose that indicated that *C. elegans* would not be effective as a model organism for *in vivo* efficacy studies of novel antibiotics.

Further tests need to be run employing a wider variety of bacteria and antibiotics to make any definitive statements about *C. elegans* efficacy as a model organism for antibiotic testing. Specifically, tests will be run using the more potent NQM 103 and NQM 105, using HTB as a control, as these compounds proved more effective against *E. faecalis in vitro* at lower concentrations. The concentration at which antibiotics are

toxic to *C. elegans* should also be determined by administering increasing concentrations of antibiotics to healthy *C. elegans.*

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