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Original article

Evaluation of Fumagilin-B® and other potential alternative chemotherapies against *Nosema ceranae* -infected honeybees (*Apis mellifera*) in cage trial assays

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Abstract – Fumagilin-B® is the only currently registered chemical treatment available to combat nosema disease in apiculture. Fumagillol, the basic hydrolysis product of fumagillin, two semisynthetic fumagillin analogues, and four in-house purely synthetic compounds which were designed to mimic the mode of action of fumagillin against the methionine aminopeptidase type 2 (MetAP-2) enzyme, was observed to exhibit statistically significant biological activity against *Nosema ceranae* -infected caged bees. None of these compounds were, however, as effective as Fumagilin-B®. The commercially available thymol and enilconazole also exhibited activity against *N. ceranae* , with thymol being the most promising chemical treatment other than Fumagilin-B®. High cumulative bee mortality was associated with the therapeutic dosage of Fumagilin-B® during our study, suggesting the need for continued investigation.

Fumagilin-B® / fumagillin / dicyclohexylamine / DCH / analogues / apiculture

1. INTRODUCTION

Nosema disease of honeybees, *Apis mellifera* L., is caused by infection from two distinct species of single-cellular microsporidian fungal parasites, *Nosema apis* (Zander 1909) and *Nosema ceranae* (Fries et al. 1996). Infections of *N. ceranae* have been shown to cause high levels of colony loss in some regions, while both species have been implicated as part of the pathogen complex associated with the colony collapse disorder (CCD) phenomenon (Cox-Foster et al. 2007; Martín-Hernández et al. 2007; Higes et al. 2008, 2009; vanEngelsdorp et al. 2009). Fumagilin-B® is the only currently available registered chemotherapy available to treat nosema disease in North America (Williams et al. 2011) and is reportedly allowed for use in special circumstances in Spain, the Balkans, and other parts of Europe (Stevanović et al. 2013). The commercial products (Fumagilin-B® and Fumidil-B®) both contain the therapeutic fumagillin as the dicyclohexylamine (DCH) salt. This results in both compounds (fumagillin and DCH) being administered in equimolar quantities when using either of the commercial formulations. DCH is reportedly five times as toxic to mice as fumagillin, and the reader is referred to a recent review where the relative toxicities of these two compounds are discussed in greater detail (van den Heever et al. 2014). Fumagilin-B® (and the

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equivalent Fumidil-B®) has been continuously employed to treat both *N. apis* and *N. ceranae* ever since the discovery (Hanson and Eble 1949) of fumagillin, when it was found to be effective in controlling *N. apis* infections in honeybees (Katznelson and Jamieson 1952; Bailey 1953).

Fumagillin and its analogues inhibit the formation of new blood vessels around tumors (angiogenesis), thereby limiting the blood supply to tumors and thus impeding their growth (Ingber et al. 1990). Fumagillin covalently binds to a histidine moiety (His^{231}) within the enzymatic active site of the methionine aminopeptidase type 2 (MetAP-2) enzyme (Griffith et al. 1997; Sin et al. 1997; Liu et al. 1998; Zhang et al. 2002). resulting in the irreversible opening of the spiro-epoxide group on the core cyclohexane skeleton of fumagillin. This spiro-epoxide is reportedly responsible for the biological activity of fumagillin, since opening of the epoxide by thermal decomposition leads to the loss of biological activity (Griffith et al. 1997, 1998; Kochansky and Nasr 2004).

MetAP-2 enzymes are ubiquitous in eukaryotic organisms, including the host honeybee and the disease-causative *N. apis* and *N. ceranae* (Zhang et al. 2002; Huang et al. 2013). Comparison of the MetAP-2 enzyme binding site amino acid sequences among humans and *A. mellifera* shows identical protein sequences, while sequences differ by only two amino acids when comparing *N. apis* and *N. ceranae* (Huang et al. 2013).

Numerous research efforts have been aimed at modulating the mode of action of fumagillin by synthesizing analogues that replace the alkene side chain (Han et al. 2000; Baldwin et al. 2002; Chen et al. 2009; Balthaser et al. 2011) in attempts to reduce the cytotoxicity of fumagillin while retaining its beneficial properties. The alkene side chain can be removed by basic hydrolysis, yielding the alcohol, fumagillol (Tarbell et al. 1961; Gochnauer and Furgala 1962; Assil and Sporns 1991). Chemical moieties such as carboxylic acids can then be coupled to fumagillol, resulting in new semisynthetic fumagillin analogues.

We postulated that the known mode of action of fumagillin against the MetAP-2 enzyme in humans is similar for MetAP-2 enzymes found in *N. apis* and in *N. ceranae*. As such, the core cyclohexane skeleton of fumagillin with the intact spiro-epoxide was used as a template for the design and synthesis of several semisynthetic compounds and synthetic compounds aimed at targeting the MetAP-2 enzyme in *N. ceranae*. These compounds, as well as other commercially available chemicals that reportedly exhibited activity against nosema disease, were evaluated for disease suppression and adult bee survival using cage trial assays.

2. MATERIALS AND METHODS

2.1. Reagents and materials

Trimethylsulfoxonium iodide (catalog no. T80500), *N*,*N'*-dicyclohexylcarbodiimide (catalog no. D80002), 4-(dimethylamino) pyridine (catalog no. 107700), acetyl salicylic acid (catalog no. A5376), piperonylic acid (catalog no. P49805), thymol (catalog no. T0501), carbendazim (catalog no. 378674), toltrazuril (catalog no. 34000), thiabendazole (catalog no. T8904), and enilconazole (also known as imazalil or chloramizole, catalog no. Y0000136) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Nozevit solution, a natural plant polyphenol extract, was obtained from Complete Bee (AK, USA). Fumagilin-B® was obtained from Medivet Pharmaceuticals Ltd. (High River, AB, Canada).

2.2. General synthetic methodology

Fumagillol was prepared by basic hydrolysis of Fumagilin-B® (Figure 1) according to Assil and Sporns (1991). Oxidation of alcohols to their corresponding ketone compounds was performed using established methodology (Dess and Martin 1991). The esterification of alcohols with carboxylic acids (Figure 2), as well as epoxidation of ketones (Figure 3), is described below. The identity of all the synthesized compounds was verified by high-resolution mass spectrometry on an Agilent 6224 time of flight (TOF) mass spectrometer (Table I).

2.2.1. Ester synthesis

The Steglich esterification (Figure 2) was used to prepare esters from the corresponding carboxylic acids and alcohols (Neises and Steglich 1978). The

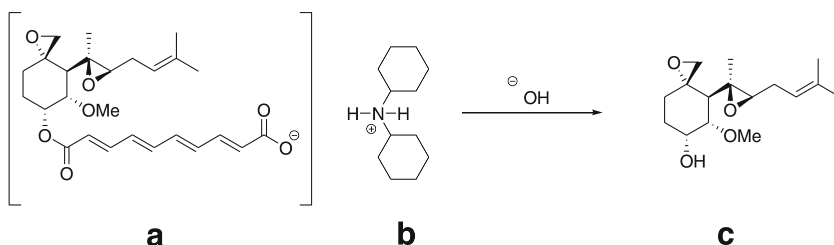

Figure 1. The commercial Fumagilin-B®, consisting of fumagillin (**a**) as the dicyclohexylamine (**b**) salt, which can be hydrolyzed to afford the alcohol, fumagillol (**c**).

general procedure involved stirring a solution of the carboxylic acid (10 mmol) in anhydrous CH_2Cl_2 (10 mL). The catalyst, dimethylaminopyridine (DMAP), was added (5 mmol), followed by drop-wise addition of the alcohol (11 mmol, 1.1 equivalent). The mixture was then cooled to 0 °C, followed by slow addition of dicyclohexylcarbodiimide (DCC; 11 mmol, 1.1 equivalent). The mixture was stirred for 5 min at 0 °C and then for 3 h at room temperature. The precipitated dicyclohexyl urea (DCU) by-product was then filtered off, and the filtrate was washed successively with 0.5 N HCl (2×10 mL) and saturated $NaHCO_3$ solution (1×20 mL). The CH_2Cl_2 fraction was dried ($MgSO_4$), concentrated in vacuo, and purified by flash chromatography on silica (230–400 mesh) using a suitable ratio of *n* -hexane and ethyl acetate as eluant.

2.2.2. Epoxide formation from ketones

A modified version of the Corey-Chaykovsky reaction was used to prepare epoxides from ketone compounds (Corey and Chaykovsky 1965; Ng 1990). shown in Figure 3. The procedure involved dissolving trimethylsulfoxonium iodide (10 mmol) into dimethylsulfoxide (15 mL) under a nitrogen atmosphere at room temperature. After stirring for 5 min, the ketone (10 mmol) was added, followed by addition of potassium tert-butoxide (10 mmol), dissolved in dimethylsulfoxide (10 mL). Stirring continued overnight at room temperature, followed by dilution with water (30 mL), extraction with CH_2Cl_2 (3×30 mL), and in vacuo concentration. Purification by flash chromatography on silica (230–400 mesh) using a suitable ratio of *n* -hexane and ethyl acetate afforded the pure epoxide.

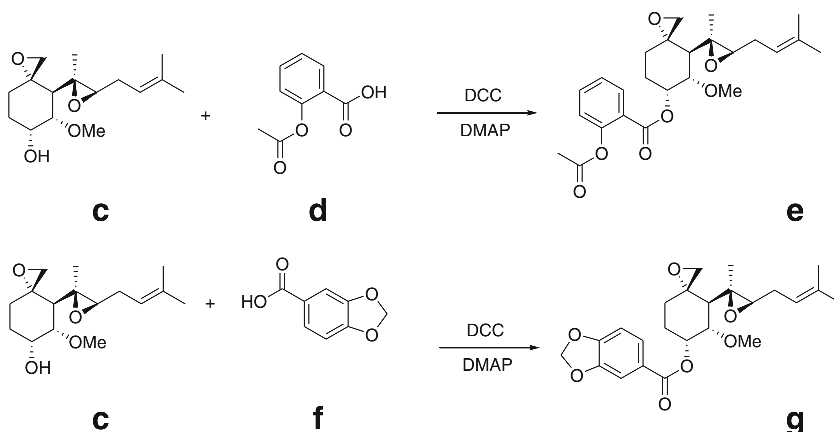

Figure 2. Coupling of fumagillol (**c**) with aspirin (**d**) or piperonilic acid (**f**), respectively, under Steglich reaction conditions using *N*,*N′*-dicyclohexylcarbodiimide (DCC) and dimethylaminopyridine (DMAP), to afford the aspirin analogue (**e**) and piperonilic acid analogue (**g**) of fumagillin.

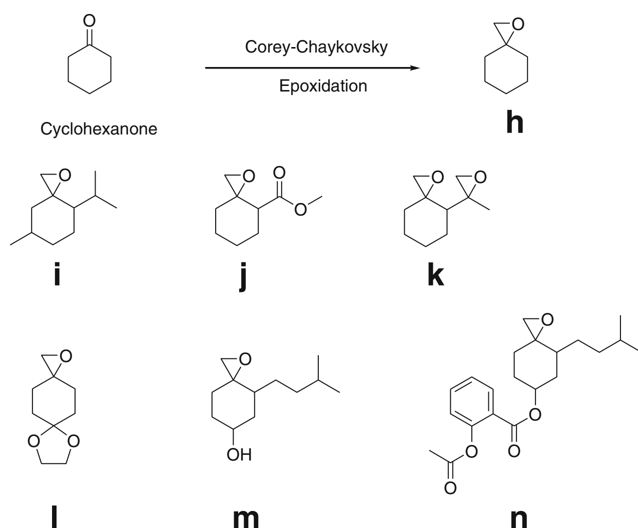

Figure 3. Purely synthetic compounds prepared by using the Corey-Chaykovsky epoxidation reaction to afford the cyclohexanone derivative (**h**), menthol derivative (**i**), and other synthetic analogues (**j**, **k**, **l**, **m**, **n**).

2.3. Cage assays—general procedures

Frames of sealed brood with newly eclosing bees (*A. mellifera*) were obtained from several colonies at Agriculture and Agri-Food Canada's Research Farm, in Beaverlodge, Alberta, Canada (55° 18′ N, 119° 17′ W). Colonies were repeatedly tested to be free from both *N. apis* and *N. ceranae*, using both light microscopic and molecular methods, described below. Frames were kept overnight in an incubator (Percival Model 136NLC9, Percival Scientific Inc., Perry, IA, USA) maintained at hive temperature (33±0.5 °C) and relative humidity (70±5 %). Adult workers were pooled and mixed from all frames, with 100 bees being added to wooden screened cages (8.0×9.5×12.0 cm I.D.) for testing. Bees were then fed 4 mL of a 60 % (*w*/*v*) of aqueous sucrose syrup for 24 h, using gravity feeders fashioned from disposable centrifuge tubes (catalog no. 93000-020, VWR International, Radnor, PA, USA).

After the initial 24-h period, each cage was mass inoculated with 5 mL of 60 % syrup solution containing 1×10^7 freshly harvested *N. ceranae* spores, prepared

Table I. Confirmation of the identity of semisynthetic and synthetic compounds that were tested against *N. ceranae* by high-resolution mass spectrometry analysis.

Compound	Found formula	Ion	Mass found	Mass calculated	Δ ppm
c	$C_{16}H_{24}O_3Na$	$[M + Na\ (-H_2O)]^+$	287.1631	287.1618	4.65
e	$C_{25}H_{32}O_7Na$	$[M + Na]^+$	467.2033	467.2040	−1.49
g	$C_{24}H_{28}O_6K$	$[M + K\ (-H_2O)]^+$	451.1512	451.1517	−1.23
h	$C_7H_{12}ONa$	$[M + Na]^+$	135.0776	135.0780	−3.49
i	$C_{11}H_{21}O$	$[M + H]^+$	169.1592	169.1587	2.72
j	$C_9H_{13}O_2$	$[M + H\ (-H_2O)]^+$	153.0913	153.0910	1.96
k	$C_{10}H_{20}NO$	$[M + NH_4]^+$	170.1531	170.1539	−4.72
l	$C_9H_{15}O_3$	$[M + H]^+$	171.1024	171.1016	4.96
m	$C_{11}H_{20}O_2Na$	$[M + Na]^+$	207.1351	207.1356	−2.02
n	$C_{25}H_{33}O_7$	$[M + H]^+$	445.2234	445.2221	3.00

from previously identified colonies of honeybees with high levels of *N. ceranae* infection. Workers from infected colonies were euthanized on dry ice; their abdomens were then removed and suspended in ultrapure water (1 mL per bee). After maceration, the crude suspension was filtered through a sieve (~0.8 mm), with spores counted according to procedures below. Suitable dilution yielded the inoculum in 60 % sucrose syrup, which was fed for 48 h to the prepared cages of bees, during which time, all the inoculum was consumed. Although mass inoculation may introduce more variability with respect to individual bee infection levels than individual bee inoculation, this method is an acceptable and efficient approach to screen a large number of compounds over several concentrations and replicates (Fries et al. 2013). We have found an inoculum spore concentration of 1×10^7 to be an effective discriminatory spore dose during previous cage trial work. It also exceeds the IC100 level for *N. ceranae*, and it also falls into the range of infection doses used in several other studies. Based on our previous work, day 17 has also been established as the day where we observed maximum infectivity. Test compounds were subsequently fed to bees in 60 % sucrose solution ad libitum for 17 days, at prescribed dosages.

Five or six replicate cages of bees were evaluated for each concentration of each compound tested. The efficacy of test compounds for suppressing *N. ceranae* spore development was assessed by determining the average number of spores per bee at 17 days post-inoculation. Cumulative bee mortality per cage over the duration of the trials was also calculated as the sum of daily bee deaths on day 17, expressed as a proportion of the initial starting population; all bees remaining on day 17 were removed for spore analysis.

2.3.1. Determination of spore levels and *Nosema* spp. identification

To determine *Nosema* infection levels in colonies, 60 adult workers were collected from peripheral frames of the brood nest, and for cage trials, 30 surviving workers were removed from each cage 17 days post-inoculation. Bees were euthanized and had their abdomens placed into a stomacher bag containing 70 % ethanol (1 mL per bee). The abdomens were then macerated for 1 min at medium speed (Seward Stomacher® 80 Biomaster, Seward Laboratory Systems Inc., Davie, FL, USA), and 6 μL of the macerate was withdrawn and loaded onto a Helber Z30000 counting chamber (Hawksley, Lancing, UK), with spores counted according to the generalized methods of Cantwell (1970) under phase contrast microscopy at 400× magnification. Samples of the remaining crude macerate were portioned into 1.5-mL microcentrifuge tubes and stored at −20 °C.

For *Nosema* spp. identification, the crude frozen macerate was thawed and vortexed, and then, 200–400 μL was centrifuged to remove the ethanol from the sample. DNA extraction was performed using the DNeasy® Blood & Tissue Kit (Qiagen®, Valencia, CA, USA). The concentration of the extracted DNA was determined spectrophotometrically (NanoDrop 2000C, Thermo Scientific, West Palm Beach, FL, USA), whereafter 50–100 ng of this DNA extract was amplified using polymerase chain reactions (PCRs).

A multiplex system that co-amplified the 16S rRNA gene of *N. apis* and *N. ceranae* (Martín-Hernández et al. 2007) as well as the honeybee ribosomal protein RpS5 gene (Thompson et al. 2007) was used within the same reaction. All PCR reactions were performed using a Mastercycler® proS thermocycler (Eppendorf, Mississauga, Canada) and utilizing the Illustra™ PuReTaq Ready-To-Go™ PCR beads (GE Healthcare Life Sciences, Baie d'Urfe, Quebec, Canada). PCR beads were reconstituted to a 25 μL final volume by adding sterile H_2O, 0.5 μL of 20-mM forward and reverse primers (a final concentration of 0.4 mM), and the DNA. All PCR products were visualized on a 2 % agarose gel and stained with SYBR® Safe DNA gel stain (Life Technologies, Carlsbad, CA, USA).

2.3.2. Testing of semisynthetic, synthetic, and commercially available compounds

In 2010, Fumagilin-B® was used to prepare three positive control solutions at concentrations of 4, 40, and 400 μM Fumagilin-B® in 60 % sucrose solution, by first dissolving the appropriate amount of Fumagilin-B® into 1.0 mL ethanol, before adding it to the 60 % sucrose solution. Concentration ranges were chosen to encompass the manufacturer's recommended dose concentration of 41 μM (105 mg fumagillin DCH salt, corresponding to 5 g of the formulation, dissolved into 4 L of sucrose syrup). The aspirin and piperonyl analogues of fumagillin (Figure 2, compounds **e** and **g**), as well as fumagillol (Figure 1, compound **c**) and the commercially available carbendazim, thiabendazole,

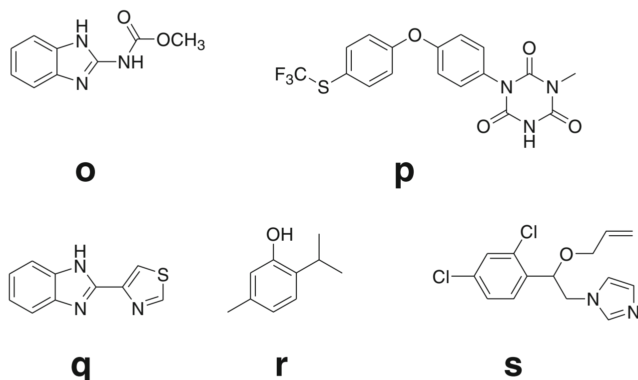

Figure 4. Commercially available compounds evaluated included carbendazim (**o**), toltrazuril (**p**), thiabendzole (**q**), thymol (**r**), and enilconazole (**s**). Nozevit, another commercially available product tested, is a bee supplement consisting of a mixture of unspecified polyphenols and other compounds.

and thymol (Figure 4, compounds **o**, **q**, and **r**), were also prepared and tested at concentrations of 4, 40, and 400 μM. A negative control consisted of only 60 % sucrose with 1 mL of added ethanol. Nozevit, sold as a bee supplement consisting of a mixture of unspecified polyphenols and other compounds, was prepared and tested as per the manufacturer's instructions. Several synthetic compounds (Figure 3, compounds **h**, **i**, **j**, and **k**) were also similarly prepared and tested.

In 2011, only a single positive control sample of Fumagilin-B® was prepared at 40 μM, along with the previously described negative control. This was done since Fumagilin-B® almost completely eliminated *N. ceranae* spores in honeybees at a concentration of 40 μM during the 2010 trials and due to the fact that we observed a significant increase in bee mortality at higher concentrations of Fumagilin-B® (Table IV). Screening of synthetic compounds at concentrations greater than 40 μM in 2011 was done in order to facilitate detection of potential biological activity, given the possibility that test compounds may not be as biologically active as Fumagilin-B®. Thus, the test concentrations of synthetic compounds in 2011 (and subsequent years) were increased to 40, 400, and 800 μM. The semisynthetic aspirin analogue (Figure 2, compound **e**) as well as the commercially available toltrazuril and carbendazim (Figure 4, compounds **p** and **o**) were also tested during 2011.

In 2012, testing was conducted using the same 40, 400, and 800 μM concentration regimen as described above for the 2011 tests. Three synthetic compounds (Figure 3, compounds **l**, **m**, and **n**), as well as the commercially available enilconazole (Figure 4, compound **s**) and aspirin by itself (Figure 2, compound **d**), was tested.

2.4. Statistical analysis

The effectiveness of compounds was evaluated by using regression models of spore counts that included compound dose as the explanatory variable. Indicators for each dose were included, compared to 0-μM negative controls as the referent, because the relationship between dose and spore count was not linear. Separate negative binomial (NB) or zero-inflated negative binomial (ZINB) regression models were run for each compound. Models were selected using the following criteria (Dohoo et al. 2009). (1) Linear regression models for spore count violated residual assumptions and were not used, despite a log transformation of spore count. (2) NB models were selected as the mean number of spore counts were not equal to the variance of Poisson models for count data (the overdispersion parameter of NB models was significantly different than 0). (3) ZINB models were selected when the Vuong test was significant ($P \leq 0.05$), indicating that there may be a separate process involved for those bees with zero spore counts. (4) For the inflated (logistic) portion of ZINB models, Akaike's and Bayesian information criteria (AIC and BIC) were used to determine the best fit for compound dose (dose indicators compared to treated

Table II. Results of the negative binomial models to assess the relationship between test compound concentration and average *N. ceranae* spore count at 17 days post-infection after feeding each compound ad libitum in 60 % sugar solution.

Compound	Number[a]	Dose (μM)[b]	IRR[c]	LRT *P* value[d]	Wald *P* value[e]	95 % confidence interval[f]
Cyclohexanone derivative (**h**)	28	0	Referent	0.03	–	–
–	6	4	0.35	–	0.02	0.14–0.87
–	6	40	0.41	–	0.06	0.16–1.03
–	6	400	0.32	–	0.02	0.13–0.81
Compound(**j**)	28	0	Referent	0.03	–	–
–	6	4	0.24	–	0.01	0.08–0.73
–	6	40	0.21	–	0.01	0.07–0.63
–	6	400	0.44	–	0.15	0.15–1.35
Compound (**k**)	28	0	Referent	<0.01	–	–
–	6	4	0.55	–	0.22	0.21–1.43
–	6	40	0.08	–	<0.01	0.03–0.21
–	6	400	0.43	–	0.09	0.17–1.13
Compound (**n**)	28	0	Referent	<0.01	–	–
–	5	40	2.56	–	0.07	0.93–7.04
–	5	400	3.49	–	0.02	1.27–9.60
–	4	800	5.64	–	<0.01	1.85–17.20
Aspirin (**d**)	43[g]	–	Dose	0.52	–	–
Compound (**i**)	46[g]	–	Dose	0.07	–	–
Compound (**l**)	46[g]	–	Dose	0.44	–	–
Compound (**m**)	46[g]	–	Dose	0.85	–	–
Carbendazim (**o**)	63[g]	–	Dose	0.67	–	–
Toltrazuril (**p**)	47[g]	–	Dose	0.09	–	–
Thiabendazole (**q**)	46[g]	–	Dose	0.12	–	–
Nozevit	46[g]	–	Dose	0.32	–	–

[a] Total number of cages of 100 bees for each dose. Spore counts for each cage represent the average number of spores per bee

[b] The dose of each compound tested compared to negative controls (0 μM)

[c] Incident rate ratio (ratio of spore counts per bee of the dose in question compared to the referent negative control dose). IRRs are not shown for compounds where the dose variable was not significantly associated with changes in spore counts (see e)

[d] Likelihood ratio test of whether or not the group of indicators for dose is significant to the model for spore count

[e] Wald tests of whether or not IRR for each individual dose compared to controls is significantly different than 1.0

[f] The 95 % confidence interval for the IRR

[g] These numbers also include 28 cages of controls

versus non-treated, lower AIC and BIC representing a better fitting model).

Likelihood ratio tests (LRTs) were used to determine if groups of concentration indicators were significantly associated with changes in spore count ($P \leq 0.05$). If the LRT was significant, Wald tests were used to compare each individual dose to the referent concentration (0 μM). The fit of NB models

Table III. Results of the zero-inflated negative binomial models to assess the relationship between test compound concentration and average *N. ceranae* spore count at 17 days post-infection after feeding each compound ad libitum in 60 % sugar solution.

Compound (model portion)[a]	Number[b]	Dose (μM)[c]	IRR/OR[d]	LRT/Wald *P* value[e]	Wald *P* value[f]	95 % confidence interval[g]
Fumagilin-B® (**a**)	28	0	Referent	<0.01	–	–
NegBin	11	4	0.36	–	<0.01	0.19–0.68
–	23	40	0.04	–	<0.01	0.02–0.07
–	11	400	1.1e-7	–	1.00	0–∞
Inflated (treated)	28	No	Referent	NA	–	–
–	45	Yes	31.82	–	<0.01	3.71–273.14
Fumagillol (**c**)	28	0	Referent	<0.01	–	–
NegBin	6	4	0.84	–	0.62	0.41–1.70
–	6	40	0.27	–	<0.01	0.12–0.59
–	5	400	0.03	–	<0.01	0.01–0.15
Inflated (treated)	28	0	Referent	0.02	–	–
–	6	4	0.00	–	1.00	0–∞
–	6	40	6.75	–	0.21	0.35–130.32
–	5	400	107.77	–	<0.01	5.58–2100.65
Aspirin analogue (**e**)	28	0	Referent	<0.01	–	–
NegBin	6	4	0.55	–	0.11	0.27–1.14
–	12	40	0.38	–	<0.01	0.22–0.64
–	13	400	0.10	–	<0.01	0.06–0.28
–	5	800	0.13	–	<0.01	0.06–0.28
Inflated (treated)	28	No	Referent	NA	–	–
–	36	Yes	6.51		0.09	0.75–56.49
Piperonyl analogue (**g**)	28	0	Referent	<0.01	–	–
NegBin	6	4	0.26	–	<0.01	0.13–0.52
–	6	40	0.28	–	<0.01	0.14–0.56
–	6	400	0.43	–	0.03	0.20–0.92
Inflated (treated)	18	No	Referent	NA	–	–
	28	Yes	7.69	–	0.08	0.79–75.94

Table III (continued)

Compound (model portion)[a]	Number[b]	Dose (μM)[c]	IRR/OR[d]	LRT/Wald *P* value[e]	Wald *P* value[f]	95 % confidence interval[g]
Thymol (**r**)	28	0	Referent	<0.01	–	–
NegBin	6	4	0.31	–	<0.01	0.16–0.62
–	6	40	0.30	–	<0.01	0.15–0.59
–	6	400	0.54	–	0.08	0.27–1.07
Inflated (treated)	28	No	Referent	NA	–	–
–	18	Yes	5.42	–	0.16	0.52–56.83
Enilconazole (**s**)	28	0	Referent	<0.01	–	–
NegBin	5	40	0.47	–	0.051	0.22–1.005
–	5	400	0.16	–	<0.01	0.07–0.34
–	3	800	0.12	–	<0.01	0.04–0.37
Inflated (treated)	28	No	Referent	NA	–	–
–	13	Yes	11.25	–	0.04	1.19–106.70

[a] Model portion refers to the negative binomial (NegBin) portion or the zero-inflated logistic (inflated) portion

[b] Total number of cages of 100 bees for each dose. Spore counts for each cage represent the average number of spores per bee

[c] The dose of each compound tested compared to negative controls (0 μM)

[d] Incident rate ratio (ratio of spore counts per bee of the dose in question compared to the referent negative control dose) for the NegBin portion. Odds ratio for the inflated portion (either indicator for each dose or for a variable is treated—yes or no)

[e] Likelihood ratio test of whether or not the group of indicators for dose is significant to the model for spore count for the NegBin model. Wald test of the group of indicators for dose for the inflated model. If treatment—yes or no—was used, refer to the Wald test in f

[f] Wald tests of whether or not IRR or OR for each individual dose compared to controls is significantly different than 1.0

[g] The 95 % confidence interval for the IRR or the OR

Table IV. Cumulative mortalities for bees infected with *N. ceranae* that were fed varying concentrations of test compounds ad libitum in 60 % sugar solution.

Treatment	Dose (μM)	Dead	Total	Mortality (%)	Mean spore count (×10^6)
Control	0	1587	2810	56.5	7.21
Fumagilin-B®	4	634	1100	57.6	4.07
	40	1634	2300	71.0	0.42
	400	939	1100	85.4	0.02
Fumagillol (**c**)	4	226	600	37.7	2.92
	40	413	600	68.8	1.00
	400	541	600	90.2	0.35
Aspirin analogue (**e**)	4	304	600	50.7	3.46
	40	267	600	44.5	2.17
	400	1084	1900	57.1	1.32
	800	441	600	73.5	0.75
Piperonyl analogue (**g**)	4	288	600	48.0	1.63
	40	276	600	46.0	1.75
	400	416	600	69.3	2.17
Compound (**h**)	4	280	600	46.7	2.50
	40	267	600	44.5	2.96
	400	332	600	55.3	2.33
Compound (**j**)	4	302	600	50.3	1.75
	40	351	600	58.5	1.50
	400	217	600	36.2	3.21
Compound (**k**)	4	264	600	44.0	3.96
	40	332	600	55.3	0.58
	400	320	600	53.3	3.13
Compound (**n**)	40	182	500	36.4	18.50
	400	427	500	85.4	25.20
	800	494	500	98.8	40.70
Thymol (**r**)	4	268	600	44.7	1.58
	40	276	600	46.0	5.38
	400	308	600	51.3	2.54
Eniconazole (**s**)	40	355	500	71.0	4.95
	400	340	500	68.0	10.80
	800	493	500	98.6	2.42

Only compounds that had a statistically significant impact on spore counts are included. One cage contained 110 rather than 100 bees

was assessed using deviance chi-squared tests ($P \leq 0.05$ indicating that the model did not fit the data) and residual analysis. The fit of ZINB models was assessed by residual analysis.

Differences in mortality for compounds significantly related to spore count were evaluated descriptively by comparing proportions of mortality for bees in all replicates of a given dose compared to controls.

All analyses were conducted in Excel 2010 (Microsoft Corporation, Redmond, WA, USA) and STATA Intercooled 13.1 (StataCorp LP, College Station, TX, USA).

3. RESULTS

Molecular identification confirmed that only *N. ceranae* was present in the inoculum used to infect our caged bees, with no *N. apis* being detected.

The results of the NB and ZINB models are shown in Tables II and III, respectively. Fumagilin-B®, as well as fumagillol (Figure 1, compound **c**), exhibited statistically significant biological activity against *N. ceranae* under our test conditions (Table III). Both the semisynthetic aspirin (Figure 2, compound **e**) and piperonyl analogues (Figure 2, compound **g**) showed statistically significant biological activity against *N. ceranae*-infected bees as well. From Tables II and III, it can be seen that four purely synthetic compounds (Figure 3, compounds **h**, **j**, **k**, and **n**), as well as the commercially available thymol and enilconazole (Figure 4, compounds **r** and **s**), also showed statistically significant biological activity against *N. ceranae*. Thymol lowered the *N. ceranae* spore count by an average of 40 % over all three test concentrations after 17 days, when compared to the control group (Table IV). The commercial formulation (Fumagilin-B®) was the only treatment in this study that was effective in eliminating *N. ceranae* spores in caged honeybees.

Cumulative bee mortalities by concentrations for those compounds shown to significantly impact spore count are shown in Table IV. It is interesting to note the higher bee mortality associated with Fumagilin-B®, when compared to the negative control. The mortality associated with Fumagilin-B® also increased with an increase in concentration.

4. DISCUSSION

All of the semisynthetic and pure synthetic compounds tested were chosen for ease of preparation using readily available starting materials that were preferentially naturally occurring compounds possessing antifungal properties. Starting materials were also selected to contain either an alcohol or ketone functional group, located on a cyclohexane moiety, which mimics the core structure of fumagillin. The alcohols and ketones were converted into epoxides, using the described methodologies. Both semisynthetic derivatives of fumagillol (Figure 1, compound **c**), namely, the aspirin and piperonylic acid analogues (Figure 2, compounds **e** and **g**), were observed to exhibit statistically significant, although lower, activity against *N. ceranae*-infected bees in cage trial assays when compared with the Fumagilin-B® positive control (Table III). Fumagillol itself also exhibited statistically significant but lower biological activity than Fumagilin-B® (Table III), which is consistent with reports citing lower biological activity of the alcohol (Gochnauer and Furgala 1962). Although the aspirin analogue (Figure 2, compound **e**) was observed to be biologically active against *N. ceranae*, aspirin by itself did not exhibit any statistically significant activity against *N. ceranae*-infected bees (Table II), even though it does possess antifungal properties.

Four of the purely synthetic compounds (Figure 3, compounds **h**, **j**, **k**, and **n**) were also observed to exhibit statistically significant activity against *N. ceranae*, though inferior to that of Fumagilin-B® (Tables II and III). One of these compounds was the simple cyclohexanone derivative (Figure 3, compound **h**), which was previously synthesized and tested for activity against the MetAP-2 enzyme, with only trace amounts of biological activity being observed (Arico-Muendel et al. 2009). Of the six commercially available compounds (including Nozevit) tested (Figure 4, compounds **o**, **p**, **q**, **r**, and **s**), only thymol and enilconazole (Figure 4, compounds **r** and **s**) were observed to be biologically active against *N. ceranae* (Table III). Thymol lowered the *N. ceranae* spore count by an average of 40 % over all three test concentrations after 17 days, when compared to the control group. Similar beneficial effects were previously described for thymol, where *N. ceranae*-infected bees treated with 0.12 mg g^{-1} thymol in candy (85 % icing sugar, 10 % honey, 5 % water, and 3.2 μL g^{-1} ethanol) were reported to have only 8.8 % of the amount of spores after 25 days, when compared to a control group (Maistrello et al. 2008). Thymol, when fed to *N. ceranae*-infected bees in a 0.1 mg g^{-1} concentration in either a 50 % (*w*/*v*) sugar syrup or in a candy form (85 % icing sugar, 10 % honey, 5 %

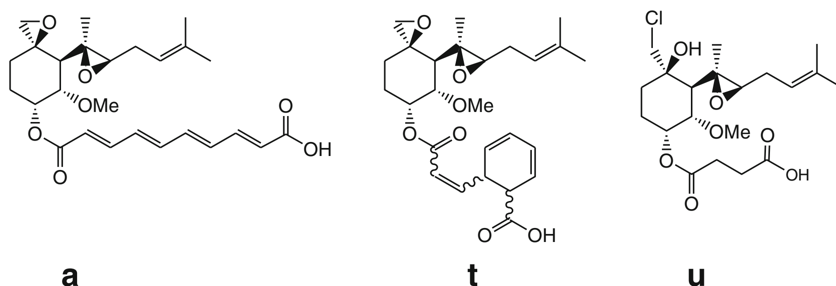

Figure 5. Comparison of the chemical structures of the biologically active fumagillin (**a**), UV-decomposed fumagillin diastereomeric compounds (**t**), and the naturally occurring chlorohydrin, ligerin (**u**), which is reportedly equally or more biologically active against osteosarcoma cell lines than fumagillin, in spite of the fact that the intact spiro-epoxide (which is reportedly responsible for biological activity of fumagillin analogues) is not present in this compound.

water, and 3.2 μL g^{-1} ethanol), reduced the *N. ceranae* spore counts by 50 % after 25 days when compared to a control group (Costa et al. 2010). while the life span of thymol-treated bees also increased by 3 days. Thymol is registered in Canada under the trade name of Thymovar™ (registration no. 29747) and is sold as cellulose-impregnated thymol fumigant for use against *Varroa destructor* (Anderson and Trueman 2000) infestations. In the EU and the USA, thymol is sold as a fumigant gel (Apiguard®), or as a combination with menthol, camphor, and eucalyptol, as an impregnated wafer (Apilife Var®). It would be interesting to establish whether thymol applied as a fumigant may be providing some secondary protection against nosema disease, compared to it being applied orally.

A recent study on the naturally occurring ligerin (Figure 5, compound **u**) found that it was equally or more active against osteosarcoma cell lines than fumagillin. Ligerin is structurally very similar to fumagillin, except for the shortened side chain and the replacement of the spiro-epoxide by a chlorohydrin moiety. This is interesting, since the spiro-epoxide on the cyclohexane skeleton of ligerin is opened (Blanchet et al. 2014). which should result in the loss of biological activity (Assil and Sporns 1991). Conversion of fumagillin, as well as and the other synthetic biologically active compounds observed in our study, to chlorohydrin compounds could perhaps result in increased biological activity against *N. ceranae*.

However, a major complication in developing new fumagillin analogues for apicultural usage based on their mode of action against the MetAP-2 enzyme is still the presumably low selectivity of fumagillin and other analogues between the host honeybee and *N. ceranae* MetAP-2 enzyme (Huang et al. 2013). Further research into the identification of other enzymes that may be present in *N. ceranae* is desirable. This might lead to the discovery of enzymes that may be crucial for the proliferation of the *Nosema* spp., while ideally not affecting the host honeybee. If such enzymes could be identified, it could afford different biological targets which could be pursued in future chemotherapy research.

The increased mortality associated with Fumagilin-B® usage requires further investigation, as Fumagilin-B® contains not only fumagillin but also the reportedly genotoxic and tumorigenic DCH. The effect of DCH on bee mortality is examined in a succeeding study (van den Heever et al. 2015).

5. CONCLUSIONS

Four purely synthetic compounds prepared for this study were found to exhibit statistically significant biological activity against *N. ceranae*, although none were as effective as Fumagilin-B®. Fumagillol and the semisynthetic aspirin and piperonyl fumagillin analogues also exhibited statistically significant activity against *N. ceranae*, but again, none of these were as effective as

Fumagilin-B®. Among the commercially available compounds evaluated, only thymol and enilconazole were found to exhibit activity against *N. ceranae*. The high bee mortality related to enilconazole usage makes this compound less attractive for commercial purposes; however, thymol is a commercially available product that might be more useful in controlling nosema disease.

Although no resistance to Fumagilin-B® has been reported in apiculture for either *N. apis* or *N. ceranae*, there has been a report of resistance being observed to the usage of Fumidil-B® for an unidentified *Nosema* spp. infecting the diamondback moth, *Pluetella xylostella* L. (Idris et al. 2001). Based on this report, it could be speculated that resistance to fumagillin may develop in apiculture in future, owing to the prolonged usage of fumagillin against nosema disease, dating back almost to its discovery in the early 1950s. Research into the development of new chemical treatments against nosema disease in apiculture is therefore of utmost importance, given that Fumagilin-B® is currently the only effective chemical treatment available.

Evaluation de la Fumagilin-B® et d'autres chimiothérapies alternatives potentielles contre des abeilles infectées par *Nosema ceranae* dans des essais en cage

***Apis mellifera* / fumagilline / dicyclohexylamine / DCH / analogues / apiculture / efficacité du traitement**

Käfigversuche zur Beurteilung von Fumagilin-B und anderer, potentiell alternativer Chemotherapien bei Nosema-infizierten Honigbienen (*Apis mellifera*)

Fumagilin-B® / fumagillin / dicyclohexylamin / DCH / analogue / Bienenhaltung

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