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Y. L. Chow *et al.* Enhanced Berberine Sensitivity in *nhr-8* RNAi *C. elegans*

Research Article

Knockdown of the NHR-8 Nuclear Receptor Enhanced Sensitivity to the Lipid-Reducing Activity of Alkaloids in *Caenorhabditis elegans*

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Received April 11, 2014; Accepted June 12, 2014

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Abstract

Caenorhabditis elegans is a versatile, whole-organism model for bioactivity screening. However, this worm has extensive defensive mechanisms against xenobiotics which limit its use for screening of pharmacologically active compounds. In this study, we report that knockdown of *nhr-8*, a gene involved in the xenobiotic response, increased the worm's sensitivity to the lipid-reducing effects of some isoquinoline alkaloids, especially berberine. On the other hand, crude extract of rhizome and cultured cells showed enhanced biological activity compared to the pure alkaloids in wild type worm, but this enhanced activity was not detected in *nhr-8* RNAi worm, suggesting that some components in cell extracts might interfere with the defense response in this worm. The possibility of using *C. elegans* as a model for screening bioactive chemicals is discussed.

Keywords:

Detoxification response, isoquinoline alkaloid, bioactivity screening, nuclear hormone receptor-8 (NHR-8), *Caenorhabditis elegans*

Nuclear receptors (NRs) are a diverse class of transcription factors that mediate hormonal signaling processes in vertebrates and insects, and are known to extend beyond the direct transduction of endocrine signals to include responses to a variety of signaling molecules (including xenobiotics), participation in multiple signal transduction pathways, and regulation of diverse physiological and developmental processes. While many of the mechanisms by which ligand-regulated, hormone-responsive NRs activate or repress the transcription of target genes have been well characterized, little is known about the cognate ligands of the remaining orphan NRs. [1] The *C. elegans* genome sequence contains 284 confirmed or predicted NR genes, which is over 5-fold more than the number found in the human genome, [2] and a few of them exhibit conserved physiological functions across taxa.

Under natural conditions, *C. elegans* is exposed to a wide range of chemical assaults found in soil. As a result, it must be able to efficiently detoxify organic chemicals for survival. Recent studies have identified many of the genes that encode sensors and enzymes that the worms may use in their xenobiotic responses such as a large number of four main classes of detoxification enzymes including cytochrome P450 (CYP), short-chain dehydrogenases (SDR), UDP-glucuronosyl or glycosyl transferases (UGT), and glutathione-S-transferases (GST). [3] Most studies on the mechanism of detoxification in *C.*

C. elegans have attempted to circumvent its xenobiotic resistance in screening for more effective potential nematicides among toxic compounds, including plant secondary metabolites.

nhr-8 is a gene that encodes a nuclear hormone receptor which is involved in the xenobiotic resistance response of *C. elegans*. Although the actual target genes of *nhr-8* have not yet been identified, the precedent PXR (pregnane X receptor) and CAR (constitutive androstane receptor) activities in vertebrates suggest that NHR-8 may regulate the expression of the cytochrome P450 genes in *C. elegans*. The toxin sensitivity of *nhr-8* is specific, since *nhr-8* RNAi worms were found to be more sensitive than wild-type worms to the toxins cholchicine and chloroquine, but not to the pathogenic bacterium *Pseudomonas aeruginosa*. [4]

Plant alkaloids have been suggested to function in the defense against herbivores and pathogens. Therefore, they are likely to induce a xenobiotic response and consequently their bioavailability might be reduced in vivo. Accordingly, the isoquinoline alkaloids, berberine and sanguinarine (Fig. 1A), were also expected to induce a xenobiotic response in these worms. Previously, the genome-wide response of the worms to berberine and sanguinarine treatment was investigated by a microarray analysis and the results indicated that defense-response and detoxification genes including *F08G5.6* (a defense-response gene), *cyp-35C1*, *gst-5*, *ugt-21*, and *ugt-25* were up-regulated (data not shown).

Although *nhr-8* RNAi worms have been used to investigate their sensitivity to toxic compounds that act as nematicides, there have been no studies on the response of *nhr-8*

RNAi worms to the bioactivities of the compounds being applied. In previous experiments with wild-type N2 worms, we found that treatment consistently yielded detectable lipid-reducing activity through Oil Red O staining at 500 μ M for berberine and 25 μ M for sanguinarine, but lower concentrations, such as 50 μ M berberine and 10 μ M sanguinarine, were less effective. [5] In an attempt to enhance the sensitivity of the worm screening system and to minimize the effective concentration of test compounds needed for bioactivity assays, the detoxification response in *C. elegans* was impeded by knocking down *nhr-8* and treating *nhr-8* RNAi worms with lower alkaloid concentrations to check if this could increase the alkaloid bioavailability in these worms. Next, we investigated if the increased sensitivity to alkaloid treatment could be due to higher bioavailability in the worms by analyzing the accumulation of alkaloids in the worms. Differences in the induction of detoxification genes between an alkaloid-treated control and *nhr-8* RNAi worms were also analyzed.

Materials and Methods

Chemicals and reagents. The following materials were used in the experiments:

berberine sulfate (Tokyo Chemical Industry Co., Ltd.), sanguinarine chloride (Sigma-Aldrich), palmatine chloride (Mitsui Petrochemical Industries), coptisine chloride (Wako Pure Chemicals, Osaka, Japan), magnoflorine (a gift from R. Nishida, Kyoto University), and columbamine (prepared in our laboratory [6]). *Coptis* rhizomes were purchased from a local market, and a *Coptis japonica* cell culture [7] was established and maintained routinely in our laboratory. All other reagents were purchased from Wako Pure Chemicals.

Coptis rhizome and cultured C. japonica cell extracts. Ten g fresh weight of *C. japonica* (156-S) cells and 10.0 g of *Coptis* rhizome slices were soaked in 100 ml methanol for 48 h, respectively. The filtered extracts were concentrated using a rotary evaporator and dissolved in distilled water for a bioassay and analysis by LC-MS.

Nematode strains. Wild-type: N2 (Bristol) were maintained on nematode growth media (NGM) at 20°C according to standard culture methods. [8]

Oil Red O staining. Two-day-old worms were treated with and without alkaloids (as a control) for 24 hours. About 200-300 worms were collected and washed three times with 1X PBS pH 7.4 buffer. Oil Red O staining was performed as previously reported. [9] The results were verified by reproducibility in at least two of three independent experiments.

RNA interference. An RNAi feeding method [10] was used. Details are described in the Supplemental Information.

Quantitative RT-PCR. Total RNA was extracted with Sepasol-RNA I Super G (Nacalai Tesque), purified with an RNeasy Mini Kit (Qiagen), and reverse-transcribed into cDNA using SuperScript III reverse transcriptase (Invitrogen) with oligo(dT) primer. cDNA (final concentration of 500 pg μl^{-1}) was subjected to qRT-PCR analysis using the CFX96 Real-Time PCR System (Bio-Rad Laboratories, Inc.) with IQ SYBR Green Super Mix (Bio-Rad). The conditions for the PCR reactions and the primer sequences are listed in the Supplemental Information.

LC-MS analysis of the accumulation of alkaloids and their metabolites in worms. Worms were washed with 0.1% SDS solution and then in M9 buffer to remove compounds stuck to the worm cuticle. After washing, worms were homogenized in 2X lysis solution (100 mM KCl, 20 mM Tris pH8.3, 0.4% SDS, 120 $\mu\text{g}/\text{ml}$ proteinase K) with coptisine chloride added as an internal standard. The details of the LC-MS analysis parameters and quantification are described in the Supplemental Information.

LC-MS analysis of Coptis rhizome and cultured cell extracts. The details of LCMS analysis parameters and quantification are described in the Supplemental Information.

132 *Statistical analyses.* The results are expressed as the mean $\pm$ standard deviation.

133 Comparisons between groups were performed by analysis of variance (ANOVA) and
134 Tukey test. $p < 0.05$ was considered statistically significant.

136 **Results**

137 *RNAi knockdown of *nhr-8* enhanced the lipid-reducing activity of berberine*

138 In wild-type N2 worms, 500 μ M berberine and 25 μ M sanguinarine showed consistent
139 lipid-reducing activity with Oil Red O staining, and lower concentrations such as 50 μ M
140 berberine and 10 μ M sanguinarine were less effective. [5] Since the detoxification process
141 might reduce the effectiveness of alkaloids, we knocked-down *nhr-8*, a nuclear hormone
142 receptor gene that is involved in the xenobiotic resistance response, with RNAi and
143 examined the worm's susceptibility to alkaloid treatment (Supplemental Fig. 1). *nhr-8*
144 RNAi worms showed significantly greater lipid reduction ($p < 0.05$) at lower concentration
145 of berberine (50 μ M) than control worm without RNAi treatment after treatment for 24
146 hour (Fig. 1B, 1C, Supplemental Fig. 2), while control RNAi worms also showed lipid
147 reduction with 10 μ M sanguinarine. These results also indicated a difference in the
148 enhancement of alkaloid sensitivity in *nhr-8* RNAi worms.

150 **nhr-8* RNAi worms accumulated more berberine and sanguinarine at low dosage*

151 To clarify the mechanism of the enhanced lipid-reducing activity of alkaloids in *nhr-8*
152 RNAi worms, we investigated the metabolites of alkaloids in worms. Recent studies have
153 reported that berberine is metabolized into four major compounds: berberrubine (m/z 322),

thalifendine (m/z 322), demethyleneberberine (m/z 324) and jatrorhizine (m/z 338) in rat plasma, human liver microsomes, and rat and human urine. [11-14] While fewer metabolites have been reported for sanguinarine due to its cytotoxic property, dihydrosanguinarine (m/z 334), the main metabolite, and some metabolites such as m/z 334 after ring cleavage, m/z 320 after successive *O*-demethylation and m/z 336 after ring-cleavage of m/z 334 were also detected in human, rat and pig liver microsomes. [15,16]

Using these metabolic data for berberine and sanguinarine as a reference, we analyzed the alkaloid metabolites in treated worms using LC-MS (Fig. 2A, 2B, Supplemental Fig. 3). Since preliminary experiments using wild-type worms did not show a distinct peak difference for cell extract or culture medium between 6 h- and 24 h-treatment, we analyzed *nhr-8* RNAi worms after treatment for 24 h for the measurement of lipid reduction. In berberine treatment, only berberine was detected and no other molecular ion peak that corresponded to any of the 4 major metabolites (berberrubine, thalifendine, demethyleneberberine and jatrorhizine) or glucuronide conjugates was found. Thus, the change in berberine content was analyzed in an *nhr-8* RNAi experiment. On the other hand, for sanguinarine treatment, sanguinarine was metabolized into several components, and the content of metabolites with m/z 334, m/z 336 were analyzed to estimate the alkaloid availability in worm.

Although we expected that the *nhr-8i* worms would accumulate more alkaloids, cellular alkaloid contents considerably fluctuated by experiments and some increases only detected at low dosages, i.e., at 50 μ M berberine and 10 μ M sanguinarine (*p*-value about 0.3 by Student's t-test). These increases were not detected at high dosages, i.e., at 500 μ M

berberine (Fig. 2C) and 25 μ M sanguinarine (Fig. 2D). While it is unclear why *nhr-8* RNAi worms accumulated less alkaloid than RNAi control worms at high dosage, other orphan receptor genes involved in xenobiotic metabolism in *nhr-8* knockdown worm might enhance the detoxification pathways at high dosages.

The major detoxification response genes induced by berberine are mostly independent of NHR-8

To characterize the enhanced lipid-reducing effects of berberine in *nhr-8* knockdown worms, we next analyzed the expressions of major detoxification genes that were up-regulated after berberine treatment in wild-type worms (data not shown). Unexpectedly, qRT-PCR results showed a similar high expression of detoxification genes after treatment with berberine in *nhr-8* knockdown worms (Fig. 3). Furthermore, the induction of detoxification defense genes was found to increase with an increased concentration of alkaloids in both control and *nhr-8i* worms. Sanguinarine had similar inducing effects on detoxification genes as berberine.

The *C. elegans* Mediator subunit MDT-15, a transcriptional coregulator in RNA polymerase II-dependent transcription, has been reported to play a role in integrating metabolic responses to ingested materials such as nutrients and xenobiotic compounds. Taubert et al. [17] reported that the expressions of *CYP35C1*, *gst-5*, and *ugt-25*, among several other detoxification genes, are MDT-15-dependent and *nhr-8* was dispensable for the MDT-15-dependent expression of these genes. Their findings correlated with our qRT-

PCR results in that the induction of major upregulated detoxification genes was independent of *nhr-8*.

nhr-8 RNAi worms survived during treatment for 24 hours, while the lipid-reducing activity of alkaloids was more enhanced. Increased expression levels of detoxification genes after treatment with berberine and sanguinarine in *nhr-8* RNAi worms could play a role in the worm's defense against these alkaloids, while *nhr-8* RNAi worms were more sensitive to some alkaloids such as cholchicine and chloroquine [4].

Effects of rhizome and cultured cell extracts on lipid accumulation in C. elegans

The greater sensitivity of *nhr-8* RNAi worms to berberine suggested that we should investigate the effects of extracts of *Coptis* rhizomes and cultured *Coptis japonica* cells, which might mimic the results of berberine in *nhr-8* RNAi worms. The cell extracts, which contain berberine, palmatine, coptisine, columbamine, magnoflorine and some unknown peaks with m/z 336, 322, 338 and 324 (Supplemental Fig. 4), were adjusted to contain about 100 μ M berberine and fed to the worms. The rhizome and cultured cell extracts showed stronger lipid-reducing effects in the RNAi control worms than berberine, whereas the effects of the cell extract relative to that of berberine were weaker in *nhr-8* RNAi worms than in RNAi control worms (Fig. 4B). Whereas extracts of rhizome and cultured cells contain several other isoquinoline alkaloids with berberine, these alkaloids such as palmatine and coptisine were also less effective on the lipid-reduction in control worm, suggesting that other type of metabolites in cell extracts would enhance the sensitivity. Measurement of *nhr-8* expression by RT-PCR indicated that the effects of cell extracts

were not due to a change in *nhr-8* expression (data not shown). The investigation of chemicals that might mimic the suppression of *nhr-8* expression is underway.

Discussion

Previously, we have shown that *C. elegans* is a versatile, whole-organism probe for screening the bioactivities of isoquinoline alkaloids. [5] However, this worm has extensive physical (such as a thick cuticle) and molecular (detoxification enzymes) defense mechanisms against xenobiotics. In many studies, the effective doses of test compounds in the worm were often several orders of magnitude higher than those in a mammalian cell culture. [18, 19] These factors limit the usage of the worm for screening the bioactivity of pharmacologically active molecules.

In this study, *nhr-8* knockdown worms were found to be more susceptible to the lipid-reducing effects of some isoquinoline alkaloids, such as berberine, palmatine and coptisine (Figs. 1 and 4). The increased sensitivity of *nhr-8* RNAi worms to alkaloid treatment suggests that a detoxification mechanism may be impaired. Although we found that *nhr-8* RNAi worms were susceptible to the lipid-reducing effects of several isoquinoline alkaloids, the degree of this sensitivity may vary due to the specificity of both the *nhr-8* receptor and the alkaloid's target. Berberine has been reported to be a substrate of the multidrug membrane transporter P-glycoprotein (P-gp) and is readily effluxed, which would result in low bioavailability. [20, 21] *nhr-8* knockdown could increase the accumulation of berberine (at 50 μ M), suggesting that P-gp function may be impeded. On the other hand, *nhr-8* knockdown showed less enhanced sensitivity to sanguinarine. Sanguinarine is known to

act as a P-gp-mediated multidrug resistance reversal agent, and inhibits ABC transporter activity. [22] Whereas we could not confirm the high content of sanguinarine in worm, sanguinarine could have higher bioavailability and thus is effective at a lower concentration (25 μ M) than berberine (500 μ M), as found in our previous experiment. [5]

Many pharmaceutical drugs are isolated directly from plants or are semi-synthetic derivatives of natural products. However, the pipeline of drug discovery faces technical challenges in isolating new compounds with diverse structures and complex chemistries in sufficient quantities for screening. [23] Thus, the ability to detect bioactivity at lower dosages using *nhr-8* RNAi worms (as observed with berberine) could be useful for future screening experiments in which the initial concentration of a bioactive compound may be too low to be effectively detected by high-throughput screening.

Note: Supplemental information is available on the Bioscience, Biotechnology, and Biochemistry website.

Acknowledgements

This research was supported by the Ministry of Education, Culture, Sports, Science, and Technology of Japan (MEXT) [Grant-in-Aid no. 21248013 to F. S.]. Y. L. Chow is the recipient of a MEXT Scholarship.

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347

Figure Legends

Figure 1

Effects of berberine and sanguinarine on lipid accumulation in *nhr-8* RNAi worms

(A) Molecular structures of berberine and sanguinarine.

(B) Effects of berberine and sanguinarine on lipid accumulation in RNAi control worm and *nhr-8* RNAi worms. Images show typical results from Oil Red O staining of worms after treatment with berberine at 50 μ M and sanguinarine at 10 μ M. Images shown at 20x magnification (Scale bar = 100 μ m). B – berberine, S – sanguinarine.

Images of worms treated with higher concentrations are shown in Supplementary Fig. S1.

(C) Effects of berberine and sanguinarine on lipid accumulation in RNAi control worm and *nhr-8* RNAi worms. Quantification of Oil Red O staining intensity using ImageJ. Values are the averages of 10-12 images for each compound tested and are normalized to the RNAi control sample; error bar= standard deviation; different letters indicate the statistic significance at $p < 0.05$ (ANOVA followed by Tukey's test). B – berberine, S – sanguinarine.

Figure 2

Accumulation of alkaloid metabolites in *nhr-8* RNAi worms.

(A) Liquid chromatogram of extracts from RNAi vector control (*pL4440*) and *nhr-8* RNAi (*nhr-8i*) worms treated with berberine at 50 μ M and sanguinarine at 10 μ M. B – berberine,

S – sanguinarine. Chromatogram of the internal standard and worms treated with 500 μ M berberine and 25 μ M sanguinarine are shown in Supplemental Fig. S3.

(B) Accumulation of berberine, sanguinarine and their metabolites in RNAi vector control and *nhr-8* RNAi worms. Semi-quantitative values are presented here as μ M equivalence of coptisine (internal standard). These values represent one of three independent experiments. Results were verified by reproducibility in at least two of three independent experiments.

L4– RNAi vector control, N8 – *nhr-8* RNAi worm. B – berberine, S – sanguinarine.

Accumulation of (C) berberine, (D) sanguinarine and their metabolites (m/z 334, 336) in RNAi control and *nhr-8* RNAi worms. Values are presented relative to the RNAi vector control for each concentration tested. These values represent the average of three independent experiments. error bar= standard deviation. L4– RNAi vector control, N8 – *nhr-8* RNAi worm. B – berberine, S – sanguinarine.

Figure 3

Effects of alkaloid treatment on RNAi control and *nhr-8* RNAi worms. qRT-PCR showed the expression of detoxification genes. The mRNA abundance value represents the average of three independent experiments. *pL4440* – RNAi vector control, *nhr-8i* – *nhr-8* RNAi worm. B – berberine, S – sanguinarine.

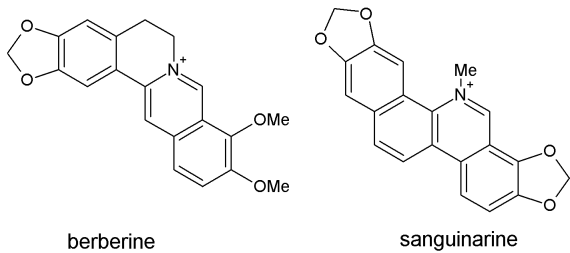
Figure 4

Effects of berberine, palmatine, coptisine, cell extracts of *Coptis* rhizomes and cultured *Coptis* cells on lipid accumulation in RNAi control and *nhr-8* RNAi worms. Oil Red O

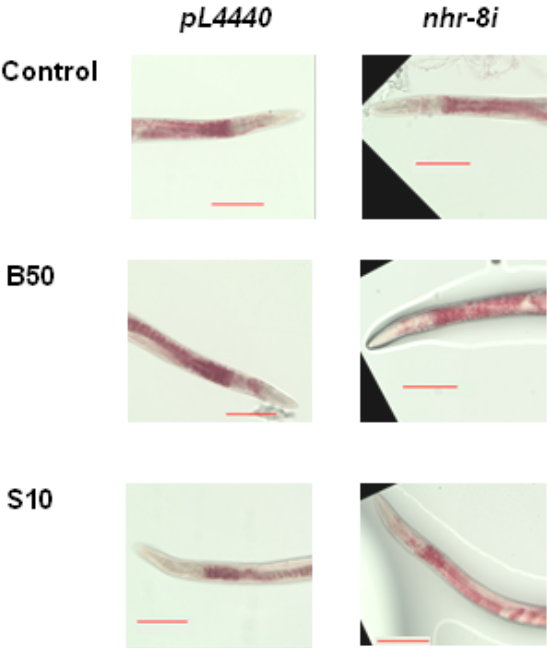
391 staining intensity quantified as the average of 10-12 images for each compound tested and
392 normalized to the RNAi control sample; error bar= standard deviation; different letters
393 indicate the statistic significance at $*p < 0.05$ (ANOVA followed by Tukey's test).
394 Berberine, palmatine, and coptisine were treated at 100 μM . Cell extract of *Coptis* rhizome
395 A contains 104 μM berberine, 30 μM palmatine, 23 μM coptisine, while that of rhizome B
396 contains 95 μM berberine, 5 μM palmatine, 5 μM coptisine and that of cultured *Coptis* cell
397 contains 105 μM berberine, 5 μM palmatine, 20 μM coptisine.

Figure 1 A, B

(A)



(B)



(C)

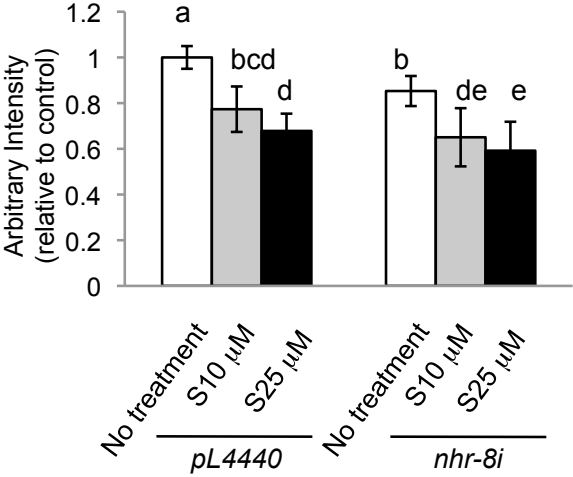
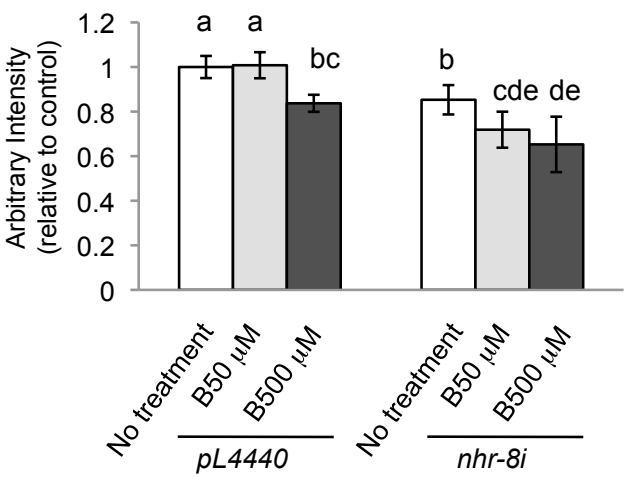
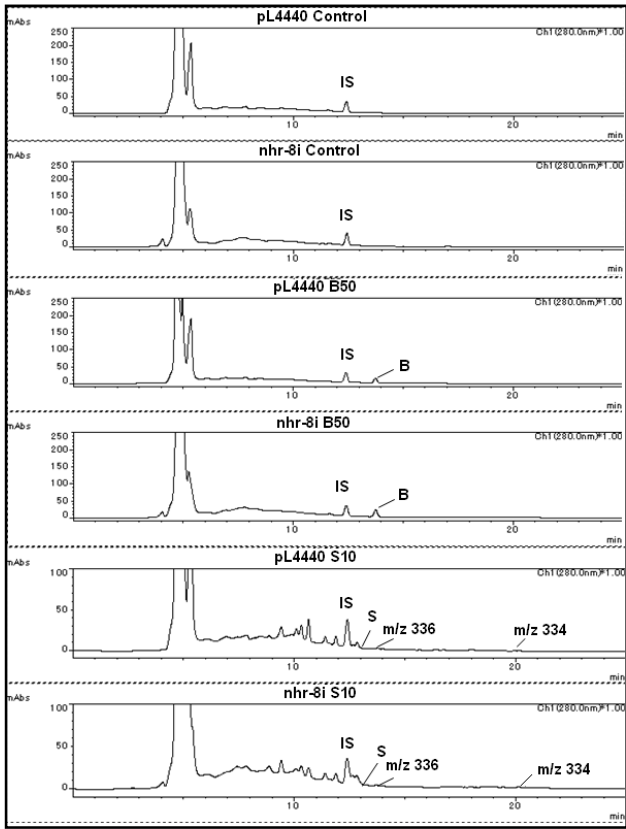
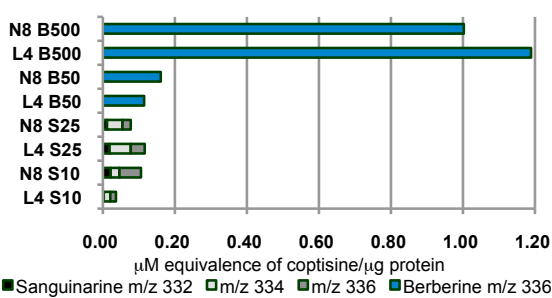


Figure 2

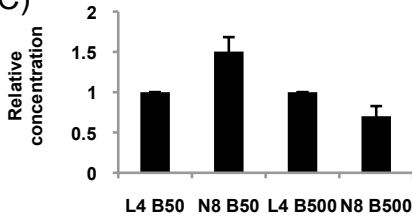
(A)



(B)



(C)



(D)

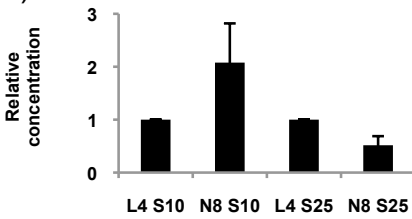


Figure 3

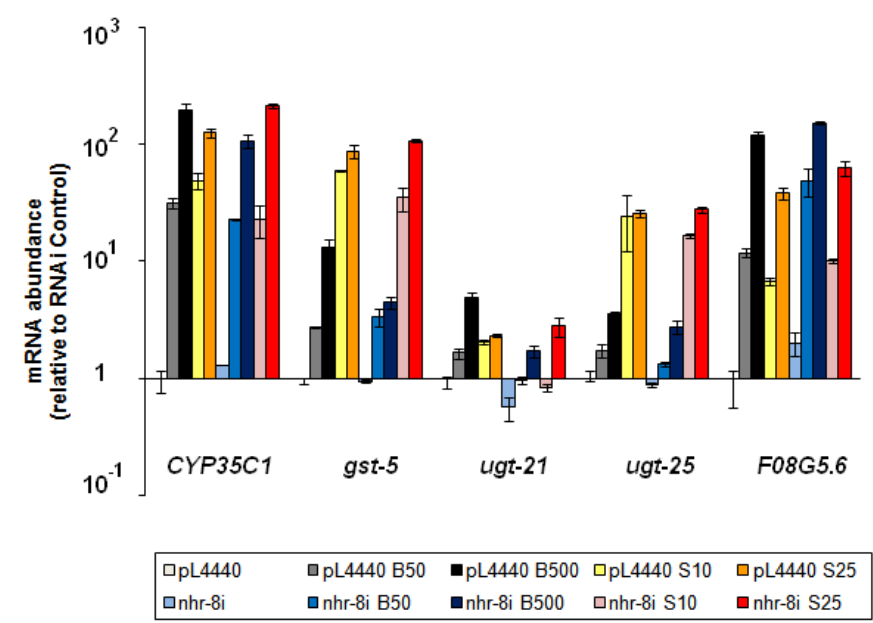
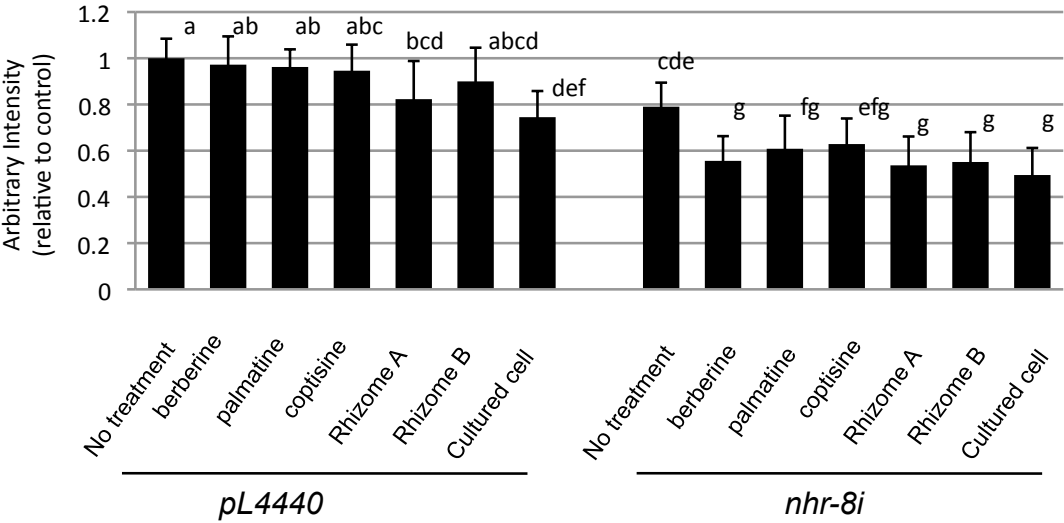
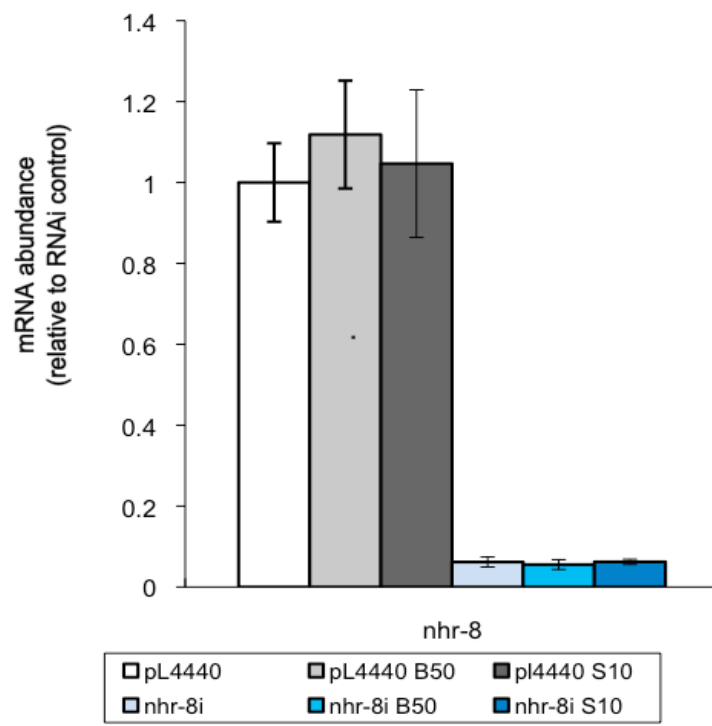


Figure 4



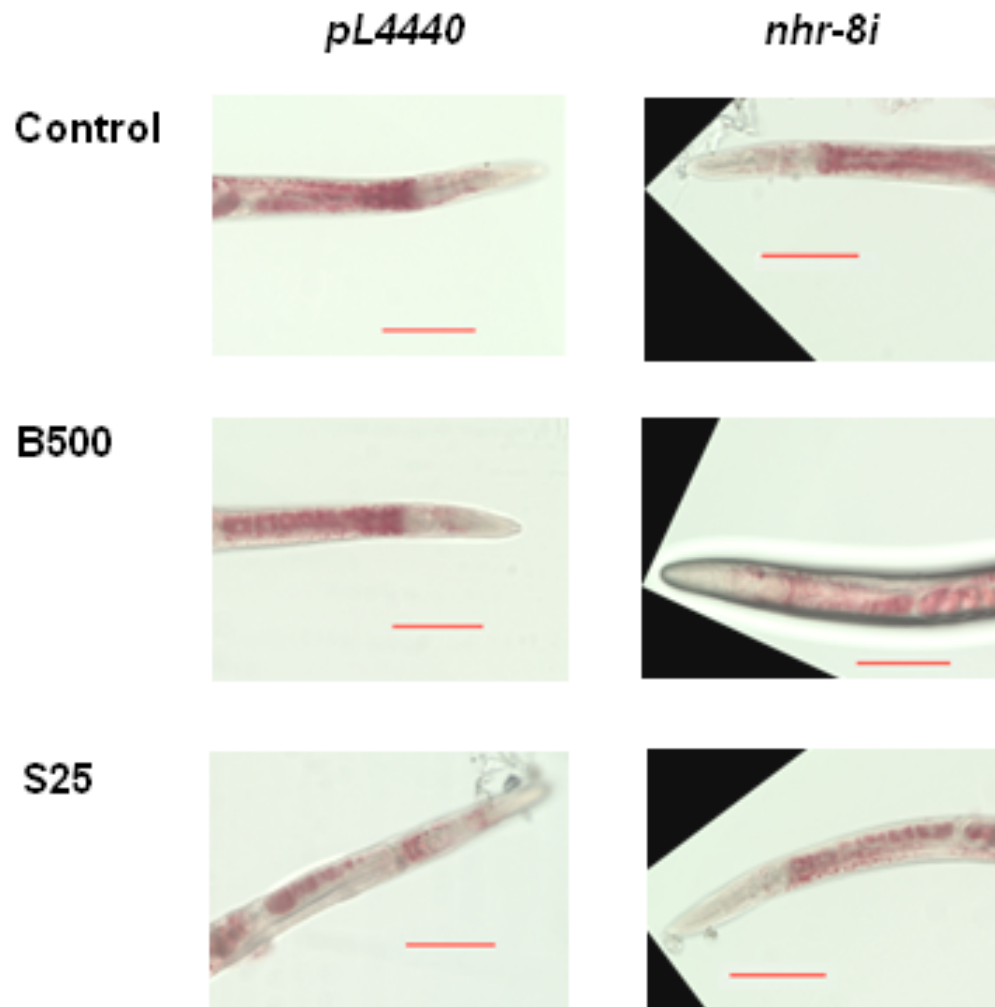
Supplemental Materials

Supplemental Figure 1



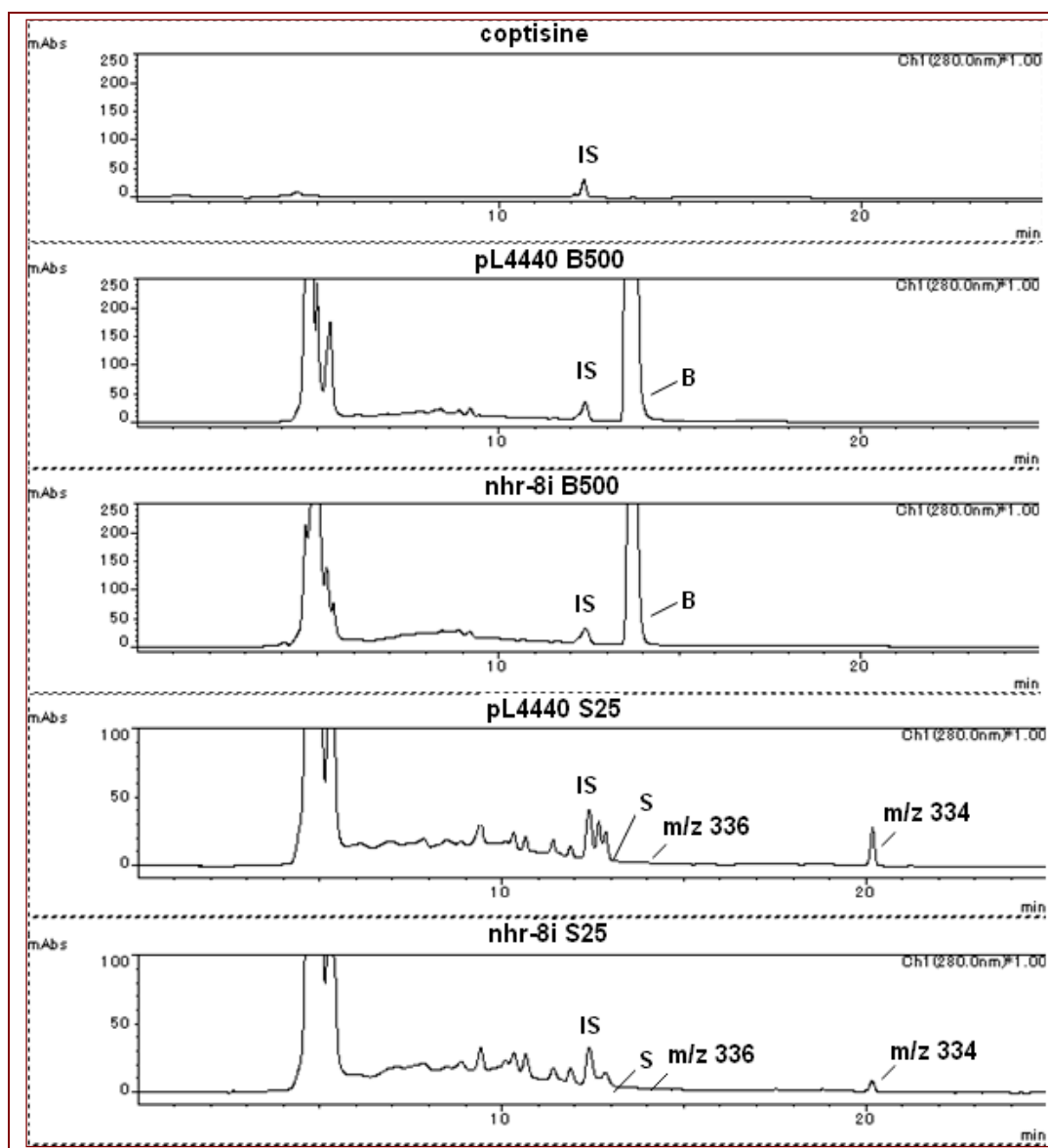
Quantitative RT-PCR of *nhr-8* transcript levels in *nhr-8* RNAi worms. mRNA abundance values represent the average of triplicate cDNA templates applied to run qRT-PCR and the results were normalized against *cdc-42* as an internal control. pL4440 – RNAi control, nhr-8i – *nhr-8* RNAi worms, B50 – berberine 50 μ M treatment, S10 – sanguinarine 10 μ M treatments.

Supplemental Figure 2



Effects of berberine and sanguinarine on lipid accumulation in RNAi control and *nhr-8* RNAi worms. Images show typical results from Oil Red O staining of worms after treatment with berberine at 500 μ M and sanguinarine at 25 μ M. Images shown at 20x magnification (Scale bar = 100 μ m). B – berberine, S – sanguinarine.

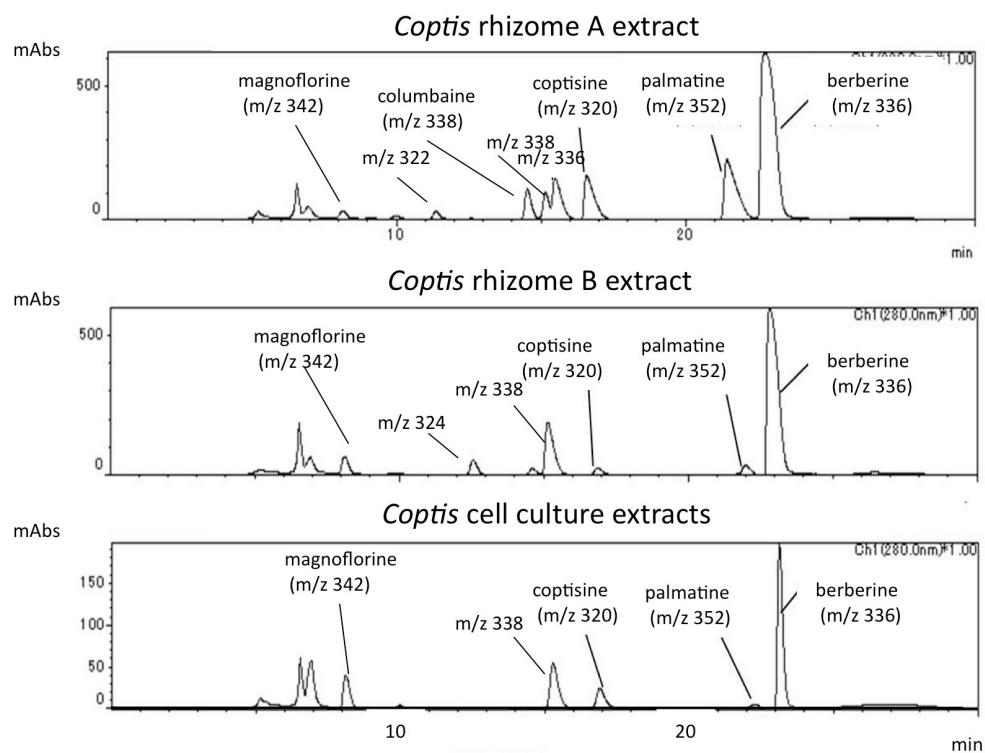
Supplemental Figure 3



Liquid chromatogram of the internal standard (coptisine), RNAi vector control

(*pL4440*) and *nhr-8* RNAi (*nhr-8i*) worm extracts. B – berberine, S – sanguinarine.

Supplemental Figure 4



Liquid chromatogram of extracts of *Coptis* rhizomes and *Coptis* cultured cells.

Materials and Methods

RNA interference

Some of the nucleotides of the coding regions of *nhr-8* complementary DNA were used for RNAi.

nhr-8 forward, 5'-ATGCCTTCGTCTTCTCCATC-3'; *nhr-8* reverse,

5'-CTCCCCAAATCCACTTTTCA-3'

nhr-8 primers were fused with Xma1 and Xho1 restriction enzyme sites and used to amplify the genes of interest. Each cDNA segment was cloned into the feeding vector pL4440 (A. Fire, Stanford University) with the respective restriction sites and transformed into HT115 bacterial cells. Colonies were screened and positive transformants were confirmed by PCR using the original primer pairs. RNAi control worms were fed bacteria carrying an empty pL4440 vector.

Quantitative RT-PCR

The conditions for PCR reactions were 95°C for 15 min, followed by 40 cycles of 95 °C for 10s, 60°C for 20s and 72°C for 20s. Melting curve analysis was performed after each run at 72°C to 95°C to check the specificity of amplification. Data were

analyzed using Bio-Rad CFX Manager (Bio-Rad) Version 1.5. The number of transcripts in a sample was determined by comparing the number of cycles (c) required for the reaction to reach a common threshold (t). The relative amount of transcript between samples was further standardized by the amplification of *cdc-42* as an internal control.

Sequences of forward and reverse primers used in quantitative RT-PCR analysis:

cyp-35C1 forward, 5'-AATTGGAGGACATCCTGTCTG-3'; *cyp-35C1* reverse,

5'-AAATACAGCTCGGCTCTTGC-3';

gst-5 forward, 5'-TCAAGCTCAACGGAAAAACC-3'; *gst-5* reverse,

5'-CCGAAGCCTTCAAGAAGTTG -3';

ugt-21 forward, 5'-AGGGAGAAATGCACAAATGC-3'; *ugt-21* reverse,

5'-CTTGCTGCAAATTCCACGTA-3';

ugt-25 forward, 5'-AAATCCGAGCCAAATGTCAC-3'; *ugt-25* reverse,

5'-TGCAAGCATATTCGCATTTTC-3';

F08G5.6 forward, 5'-GTCCCACTGTCACAAGCTCA-3'; *F08G5.6* reverse,

5'-GTTTCGACCGAGAAATCGAG-3';

nhr-8 forward, 5'- ATGCCTTCGTCTTCTCCATC -3'; *nhr-8* reverse, 5'-

CTCCCCAAATCCACTTTTCA -3';

cdc-42 forward, 5'-AGCTTCATTCGAGAATGTCC-3'; *cdc-42* reverse,
5'-CTCGAGCATTCCTGGATCAT-3'.

LC-MS analysis of the accumulation of alkaloids and their metabolites in worms

Lysis samples were incubated 60°C for 1 h and then cooled on ice. Hundred µl cold acetonitrile was added to each lysate, mixed by pipetting, and centrifuged at 800 g for 5 min. Forty µl of sample was injected for analysis by an LCMS2010 system

(Shimadzu) with the following parameters:

Column = Cosmosil 4.6 x 250 mm 5C₁₈-AR-300; Column temperature = 27°C;

Solvent A = 4.9:95:0.1 (acetonitrile/H₂O/trifluoroacetic acid (TFA));

Solvent B = 95:4.9:0.1 (acetonitrile/H₂O/TFA) using gradient elution as shown below.

<u>Time</u>	<u>Solvent</u>	<u>Flow rate (ml/min)</u>
0.00	15% B	0.7
0.15	15% B	0.7
16.75	70% B	0.7
20.00	100% B	1.0
23.00	100% B	1.0

24.00 15% B 0.7

25.00 15% B -

UV absorbance was measured at 280 nm; mass range (m/z 100 to 800) was determined in both single-ion and scan modes.

The accumulation of alkaloids and their metabolites were calculated based on the LC peak area relative to the internal standard peak of coptisine. This value is normalized to the worm protein content in each sample. Protein was extracted with 1X Sample buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 6% 2-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue, distilled water). Protein concentrations were determined by the Bradford method.

LC-MS analysis of Coptis rhizome and cultured cell extracts

Five µl of standard or 10 µl of extract was injected for analysis by an LCMS2010 system (Shimadzu) with the following parameters:

Column = TOSOH TSK-Gel ODS 80-Tm silica-based, reversed phase 4.6 x 250 mm;

Column temperature = 40°C; Flow rate = 0.5 ml/min;

Solvent A = 99.95:0.05 (H₂O/TFA);

Solvent B = 99.95:0.05(acetonitrile/TFA) using gradient elution as shown below.

<u>Time</u>	<u>Solvent B (%)</u>
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0.00	15
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15.00	35
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19.00	70
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22.00	70
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26.00	35
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30.00	35
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Peaks were identified by direct comparison with standard chemicals and alkaloid concentrations were quantified relative to the peak area of standards.