Caenorhabditis elegans as a whole organism screening system for isoquinoline alkaloid bioactivities

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Abstract

The presence of valuable bioactive molecules in plant alkaloids renders isoquinoline alkaloid biosynthesis one of the most focused targets for metabolic engineering as well as chemical synthesis. However, due to the lack of an effective, rapid and broad-based bioactivity screening tool, many of these alkaloids remained unexplored for the development of novel pharmaceutical compounds. Research into the molecular and developmental biology of *Caenorhabditis elegans* was begun in 1965 by Sydney Brenner and it has since been used extensively as a model organism. This worm model offers several distinct advantages including low running cost, ease of manipulation and short generation time which allows rapid experimentation. The completion of *C. elegans* genome sequencing revealed that many important biological processes are evolutionarily conserved between human and the worm; thus making this worm a popular model to study drug-target interaction and target validation.

In this study, *C. elegans* was used as a whole organism model to screen for biological activities of isoquinoline alkaloids. Chapter I describes the screening of isoquinoline alkaloids for lipid reducing activities. Using berberine as a reference compound which was reported to exhibit triglycerides- and cholesterol-lowering properties, worms were stained with Nile Red and Oil Red O stain to observe changes in lipid accumulation in vivo. Among the several classes of isoquinoline alkaloids tested, only berberine and sanguinarine were found to be lipid metabolism modulators, with sanguinarine showing more potent lipid-reducing activity at 20-fold lower concentration. Further investigation was carried out on the involvement of AMPK and transcription factors in the lipid metabolism pathway responsible for the lipid reduction activity upon berberine and sanguinarine treatment. Immunoblotting revealed AMPK activation in berberine- and sanguinarine-treated worms. RNAi experiments further showed involvement of AAK-2, the *C. elegans* homologs of the catalytic α-subunits of AMPK, in the lipid-reducing effect of berberine and sanguinarine. On the other hand, this effect was found to be independent on NHR-49, a hormone nuclear receptor gene that functions as key regulator of fat consumption. These results suggest that the alkaloids' lipid reducing effect predominantly affect lipid synthesis, rather than fatty acid β-oxidation.

In Chapter II, the worm model was applied to screen extracts from metabolically engineered plant cell cultures for synergistic bioactivities in plant alkaloids. As known in the case of most ethnobotanical or traditional medicine applications, it is a concoction of plant extracts that produced therapeutic effects. The lipid reducing effect was compared to that observed in single compounds. The lipid reducing effect of *Coptis japonica* cell culture extract showed higher activity than berberine at similar concentration. However, extract from an *Eschscholzia californica* cell culture required higher concentration of sanguinarine than in single compound treatment. This shows that cell extracts contain both synergistic and antagonistic components that may lead to differences in the bioactivity strength compared to single alkaloids.

The function of plant alkaloids has been suggested as defense against herbivores and pathogens, therefore they are likely to induce xenobiotic response and consequently their bioavailability might be reduced in vivo. Accordingly, the isoquinoline alkaloids, berberine and sanguinarine, were also expected to induce xenobiotics response in the worms. Chapter III describes 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*, a gene encoding a nuclear hormone receptor which is involved in xenobiotic resistance response. *nhr-8* RNAi worms were found to be more susceptible to the lipid reduction effects of some isoquinoline alkaloids, especially berberine which could reduce lipid accumulation at about 8-fold lower concentration than in wild-type worms. The *nhr-8* RNAi worms were expected to accumulate more alkaloids when the detoxification response was knocked down but the results showed that it occurred only at low dosages. It is plausible that the lower, sub-lethal alkaloids dosages would trigger the defense response genes to a moderate level that NHR-8 knockdown was effective to suppress such response and resulted in higher alkaloid accumulation in the *nhr-8* RNAi worms. Although enhanced sensitivity of the *nhr-8* RNAi worms was expected to have suppressed detoxification mechanism, the major upregulated detoxification genes were found unimpeded, except for *ugt-21* which could be a target of NHR-8.

Abbreviations

ACC	- acetyl-CoA carboxylase
AICAR	- 5-aminoimidazole-4-carboxamide ribotide
AMP	- adenosine monophosphate
AMPK	- AMP-activated protein kinase
ATP	- adenosine triphosphate
BSA	- bovine serum albumin
CYP	- cytochrome P450
GST	- glutathione-S-transferase
HMG-CoA	- 3-hydroxy-3-methylglutaryl-CoA
HPLC	- liquid chromatography-mass spectrometry
HRP	- horseradish peroxidase
IPTG	- isopropyl-β-D-thiogalactopyranoside
IQA	- isoquinoline alkaloid
KPB	- potassium phosphate buffer
mTOR	- mammalian target of rapamycin
NGM	- nematode growth medium
NHR	- nuclear hormone receptor
PBS	- phosphate buffered saline
PBST	- phosphate buffered saline with Tween-20
P-gp	- p-glycoprotein
PVDF	- polyvinylidene difluoride
qRT-PCR	- quantitative real-time polymerase chain reaction
RNAi	- RNA interference
SDS-PAGE	- sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBST	- tris buffered saline with Tween-20
TFA	- trifluoroacetic acid
Tris	- tris (hydroxymethyl) aminomethane
UGT	- uridine diphosphate glucuronosyltransferase

Introduction

Alkaloids are natural occurring, low molecular weight, nitrogenous secondary metabolites found in about 20% of flowering plant species and many among them were reported to exhibit potent pharmacological activities. There are increasingly extensive investigations on the therapeutic effects of these plant natural chemicals for drug applications. For this purpose, a rapid and broad-based bioassay system is ideal for the preliminary screen.

In this study, *Caenorhabditis elegans* was used as a screening model to detect biological activities of isoquinoline alkaloids since it has many advantageous features as preliminary screen in terms of cost, time and ease of manipulation (Corsi, 2006). It is relatively inexpensive to cultivate worms for use in the lab. Due to its small size, the worm is easy to physically manipulate and most assays can be carried out in microtitre or agar plates. Its average lifespan of three weeks is advantageous for lifespan and ageing studies. About 40% of the genes associated with human diseases have homologs in the *C. elegans* genome, making it a good model for drug development research. In fact, the popularity of *C. elegans* as a model for drug discovery is evident by the amount of published work on chemistry-to-gene, gene-to-drug screen using this nematode (Link et al., 2000; Kaletta and Hengartner, 2006; Burns et al., 2010). Genes do not work in isolation to generate particular phenotypes, rather they interact with other genes and are influenced by the environment, therefore using whole-animal invertebrate models, such as *C. elegans*, have proven to be useful in these endeavours. Besides, an intact animal has advantage over in-vitro or cell-based assays as many compounds that exhibit bioactivities fail to show efficacy when applied to whole animal (Stelling et al., 2004; Knight et al., 2007). As model organism, *C. elegans* also offers physiological advantages such as rapid reproduction cycle and transparent body wall which enable the use of in vivo fluorescence markers and stains to study processes such as axon growth, embryogenesis and fat metabolism in the worm.

In the preliminary experiment, worms were treated with isoquinoline alkaloids to screen for lipid-modulating activity. Although the mammalian regulatory pathways involving energy homeostasis are more sophisticated than those of *C. elegans*, the core mechanisms are largely conserved. Lemieux et al. (2011) also indicated the usefulness

of *C. elegans* in screening small molecules to identify new regulators of fat storage. Berberine, a benzylisoquinoline alkaloid commonly used as an antibacterial agent for gut infection and diarrhea, has recently been reported to show triglycerides and plasma cholesterol-lowering effects and improved insulin action in high-fat-fed rats. Using berberine as a reference compound, the lipid reduction effect of alkaloids in the worm was examined using staining method with Nile Red and Oil Red O. Potent lipid metabolism modulators identified from the preliminary screen were further investigated on their action mechanisms. Recent reports suggested activation of AMP-activated protein kinase (AMPK) by berberine is responsible for its lipid-reducing effect. As there are various types of AMPK activator and also various mechanisms for AMPK activation (Hawley et al., 2010), further investigation was carried out on the involvement of AMPK and transcription factors in the lipid metabolism pathway responsible for the lipid reduction of alkaloids using immunoblotting and RNA interference methods.

In traditional medicine applications, extracts that contain a mixture of active components are usually used to yield therapeutic effect. Unlike harvesting medicinal plants from the field which is limited in supply and time-consuming, one possible way to enhance production is through metabolic engineering. Metabolic engineering of plant secondary metabolites biosynthesis pathway could improve quality of metabolites by reducing undesired pathways, introducing new pathways to produce novel compounds, or by completely blocking a pathway to accumulate intermediates. In addition to screening single alkaloids, the established *C. elegans* screening system was next applied to screen for synergistic lipid modulating activity of metabolically engineered plant cell culture extracts.

Recent studies on *C. elegans* have identified many genes encoding sensors and enzymes that the worms may use in their xenobiotics responses such as a large number of four main classes of detoxification enzymes including cytochromes P450, short-chain dehydrogenases, UDP-glucuronosyl or glycosyl transferases, and glutathione-S-transferases (Lindblom and Dodd, 2006). In order 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 a gene encoding a nuclear hormone receptor which is involved in

xenobiotic resistance response, *nhr-8*. Although *nhr-8* RNAi worm had been used to investigate its sensitivity to toxic compounds that act as nematicide, there is no report on studies done to explore *nhr-8* RNAi worms' response to the bioactivities of the compounds they were treated with. Further analysis of alkaloids accumulation in the worms was done to find out if the increased sensitivity to alkaloid treatment could be due to higher bioavailability in the worms. In addition, the differences in detoxification genes induction between the alkaloid-treated RNAi control and *nhr-8* RNAi worms were also analyzed.

CHAPTER I

Screening of isoquinoline alkaloids for potent lipid metabolism modulator

Metabolic syndrome is becoming more prevalent in our modern society, posing health risks such as cardiovascular disease, stroke and diabetes. Extensive studies are being done to search for potent lipid-reducing agents as obesity is linked to the risk factors of metabolic syndrome. Currently, the drugs used clinically to control metabolic disorders are mainly synthetic compounds such as metformin, thiazolidinediones to treat insulin resistance in Type-2 diabetic patients, and lovastatin, alpha lipoic acid to treat hyperlipidemia. The search for potent lipid-reducing agents among plant natural chemicals is gaining more interest. Several natural compounds have been identified as good candidates such as catechin found in green tea (Lee et al., 2009), capsaisin in chilli pepper (Iwasaki et al., 2011), and resveratrol in grape seeds and peanut roots (Ahn et al., 2008).

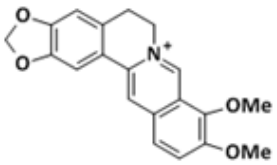
In this study, the effects of several isoquinoline alkaloids (Fig. 0-1) on lipid accumulation were examined using a vital dye, Nile Red, which stains lipid droplets in *C. elegans*. Nile Red has been widely adopted in *C. elegans* experiments (McKay et al, 2003; Ashrafi et al., 2003; Lemieux et al., 2011). However, recent studies reported that Oil Red O stain is a more accurate proxy for major fat stores in the subcellular compartments of the worm (O' Rourke et al., 2009). Thus Oil Red O was used to validate changes in lipid storage for chemical treatment that showed lipid reduction effect after the preliminary screening of chemicals with Nile Red staining.

Berberine, a benzylisoquinoline alkaloid obtained from *Berberis* (Berberidaccae) and *Coptis* rhizomes has been in use traditionally for intestinal infection based on its antibacterial property. Recently, investigation on its effect on metabolic syndromes such as obesity and diabetes are increasing. Since effects such as decrease in plasma cholesterol and triglycerides in hypercholesterolemic patients, reduced body weight and plasma triglycerides and improved insulin action in high-fat-fed rats have been reported, it was suggested as a new cholesterol-lowering drug (Kong et al, 2004). Those bioactivities of berberine were reported to be associated with the activation of AMP-activated protein kinase (AMPK) via the modulation of downstream molecules (Lee et

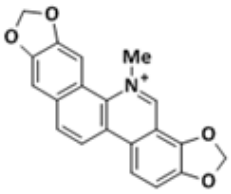
al., 2006; Hwang et al., 2009; Hawley et al., 2010); for instance, the inhibition of ACC in HepG2 cells inhibits cholesterol and triglyceride synthesis (Brusq et al., 2006). The insulin-sensitizing effect of berberine is reported to have resulted from its activation of AMPK which promoted the assembly of high molecular weight (HMW) adiponectin and increased the HMW/Total adiponectin ratio in adipocytes (Li et al., 2011).

Sanguinarine is a benzophenanthridine alkaloid present in the Papaveraceae, Fumariaceae, and Rutaceae families of plants and has been reported to exhibit antimicrobial, anti-inflammatory and antitumor activities. It is being used as antimicrobial component in oral hygiene products and animal feed additive. Sanguinarine was recently found to be a direct activator of AMPK (Choi et al., 2011), but its application as a lipid modulating agent has not been reported so far.

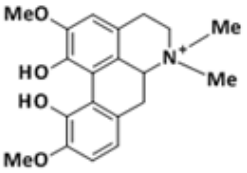
AMPK functions like a biological "fuel gauge" (Beale, 2008). It is activated under conditions that deplete cellular ATP and elevate AMP levels, such as glucose deprivation, heat shock, hypoxia, and ischemia, and also by hormones like leptin, adiponectin, catecholamine, and interleukin-6. Recent studies have demonstrated that AMPK can also be activated by other stimuli that do not cause a detectable change in the AMP/ATP ratio, like hyperosmotic stress and pharmacological agents like thiazolidinediones (TZD), metformin, and AICAR. Upon activation, AMPK phosphorylates and inactivates a number of metabolic enzymes involved in ATP-consuming pathways including acetyl CoA carboxylase (ACC), fatty acid synthase, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, mammalian target of rapamycin (mTOR), and activates ATP-generating process like fatty acid oxidation and glucose uptake (Fig. 0-2). AMPK signaling is thus considered a prime target for new therapies for metabolic disorders such as insulin resistance and type 2 diabetes (Hardie, 2011). Here, the effects of berberine and sanguinarine on lipid reduction through the lipid synthesis and fatty acid oxidation via NHR-49, a key regulator of fat consumption in *C. elegans*, were examined.



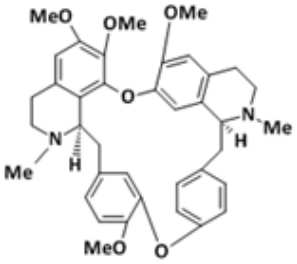
berberine



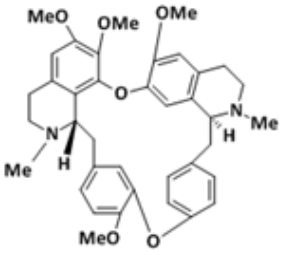
sanguinarine



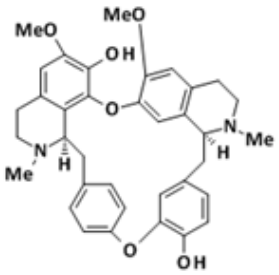
magnoflorine



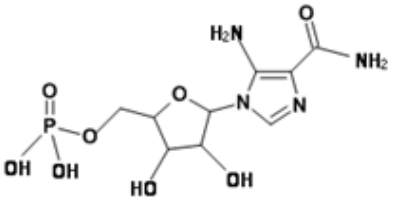
isotetrandrine



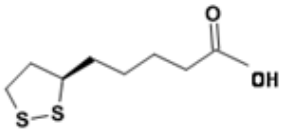
tetrandrine



aromoline



5-aminoimidazole-4-carboxamide-1-4-ribotide (AICAR)



alpha lipoic acid

Fig. 0-1 Molecular structures of test compounds

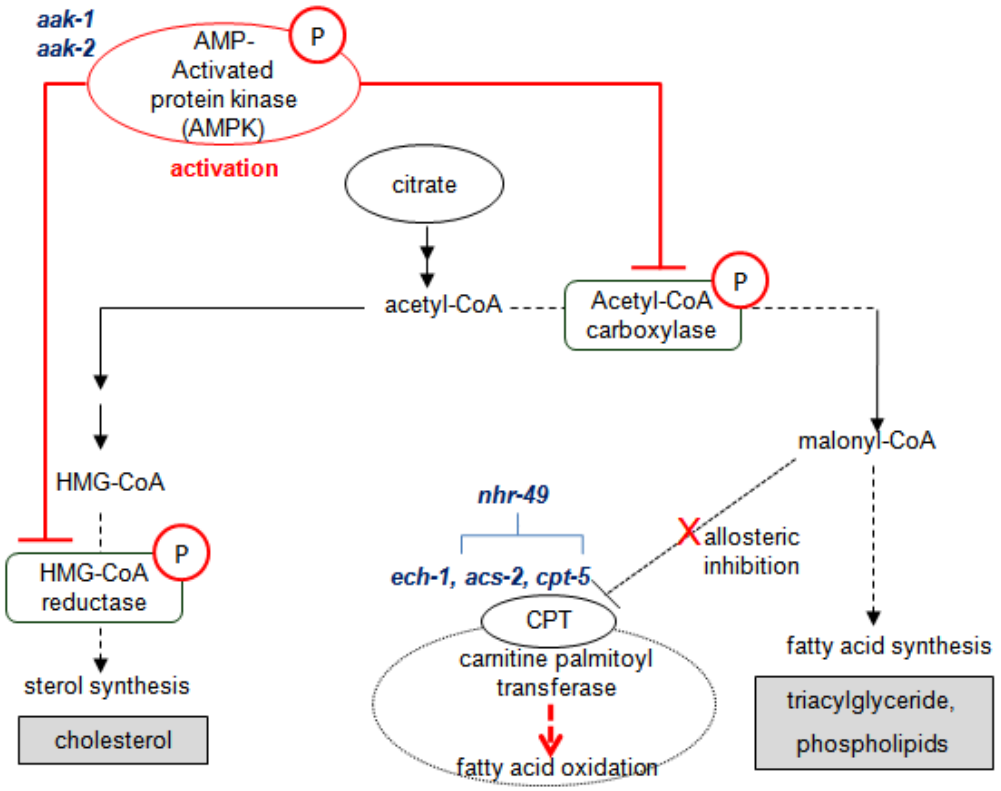


Figure 0-2 Scheme showing the effects of AMPK activation on the lipid metabolism pathway.

When AMPK is activated, it phosphorylates and inactivates several metabolic enzymes involved in ATP-consuming pathways such as acetyl-CoA carboxylase (ACC), fatty acid synthase, and 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase. The inhibition of ACC leads to the decreased conversion of acetyl-CoA to malonyl-CoA, which subsequently activates carnitine palmitoyl transferase (CPT). CPT transports long-chain acyl-CoAs into the mitochondrial matrix for β-oxidation, which eventually enters the citric acid cycle and generates ATP molecules.

The *C. elegans* genes included in this study are shown in blue italics. *aak-1* and *aak-2* are homologs of the catalytic α-subunits of AMPK in the worm. *nhr-49* encodes a *C. elegans* nuclear hormone receptor which serves as a key regulator of fat consumption by targeting several enzymes that are involved in mitochondrial β-oxidation, including *ech-1*, *acs-2*, and *cpt-5*.

Materials and Methods

Chemicals and Reagents

Berberine sulfate was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan); AICAR from Wako Pure Chemicals (Osaka, Japan); sanguinarine chloride, α-

lipoic acid and cholesterol from Sigma-Aldrich (St. Louis, MO, U.S.A.); and magnoflorine, aromoline, tetrandrine, and isotetrandrine were kind gifts from Dr. K. Iwasa (Kobe Pharmaceutical University). Phospho-Thr172-AMPK, AMPK, phospho-Ser79-ACC and ACC antibodies were from Cell Signaling Technology (Beverly, MA, U.S.A.) and horseradish peroxide (HRP)-conjugated donkey anti-rabbit IgG from GE Healthcare (Buckinghamshire, U.K.). All other reagents were purchased from Wako Pure Chemicals unless otherwise stated.

Nematode Strains

Wild-type: N2 (Bristol) were maintained on nematode growth media (NGM) at 20°C according to standard culture methods (Stiernagle, 2006).

Nematode Treatment

In the preliminary screen using Nile Red staining, 3-day-old worms were used. When larger amount of worms were required for Oil Red staining, protein and RNA extraction, 2-day-old worms were used for treatment in subsequent assays to avoid a mixed population of adult worms, eggs, and larval L1 at the end of 24h treatment.

Nile Red Staining

Synchronized 3-day-old *C. elegans* were treated with various chemicals for 24 hours on NGM medium plated with *E. coli* OP50 as a food source, 50 ng ml^{-1} Nile Red (Sigma Chemicals) and test compounds at the indicated final concentrations. Ten to 12 worms were treated in duplicate for each test compound in three independent experiments. After 24 hours of culture at 20°C, 10 to 12 worms were randomly selected for observation under bright field, and red fluorescence images were obtained using a Keyence BIOREVO BZ-9000 Imaging System (Keyence Corporation, Osaka, Japan). Hazy images were omitted and the rest were used to quantify the fluorescence intensity of lipid droplets using ImageJ software (http://rsbweb.nih.gov/ij/). The results were verified by reproducibility in at least two of three independent experiments. The figures represent the results from one experiment.

Oil Red O Staining

Two-day-old worms were treated with and without alkaloids (as a control) for 24 hours as in the Nile Red staining experiments. 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 by O' Rourke et al. (2009). After staining, 10 to 12 worms were randomly selected for observation under bright field, and images were captured using a Keyence BIOREVO BZ-9000 Imaging System. Hazy images were omitted and the rest were used to quantify the intensity of Oil Red O staining using ImageJ software. Each color image was separated into its RGB channel components and the green channel was used for further analysis following previous method by Yen et al. (2010). The results were verified by reproducibility in at least two of three independent experiments. The figures represent the results from one experiment.

RNA Interference

An RNAi feeding method (Lehner et al., 2006) was used. Part of the nucleotides of the coding regions of *aak-1*, *aak-2*, and *nhr-49* complementary DNA were used for RNAi. The following primers were used:
aak-1 forward, 5'-ATGCCTCCAAGTGGACGTTTCGATA-3'; *aak-1* reverse, 5'-CAGCAAGTAGAGCTCCAGTTACATC-3';
aak-2 forward, 5'-ATGTTTTCTCATCAAGATCGAGACCG -3'; *aak-2* reverse, 5'-TACAACTTTCCGCTAATAACCTCAGG-3';
nhr-49 forward, 5'-ATGGACCTAGTAGATCCTCTTG-3'; *nhr-49* reverse, 5'-AGAGGATGAATTGCCAATGGAG-3'.

Primers were fused with restriction enzyme sites: Xba1, Xho1 for *aak-1* and *aak-2*; Xba1, Xma1 for *nhr-49* and were used to amplify the genes of interest. Each cDNA segment was cloned into feeding vector pL4440 (A. Fire, Stanford University, CA, U.S.A.) with 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.

HT115 bacteria containing an RNAi vector or control vector were grown in Luria-Bertani (LB) broth with 25 μg ml^{-1} carbenicillin overnight at 37°C, and induced with 1

mM isopropyl-β-D-thiogalactopyranoside (IPTG) for the final 3 hours. The bacterial culture was pelleted and resuspended in the same volume of S-medium with added 1 mM IPTG and 25 μg ml^{-1} carbenicillin, and 500 μl was dispensed into each well of a 24-well plate. Two synchronized L3 larval worms were added to each well. Plates were incubated at 20°C for 3 days, during which period the worms would have laid eggs that then hatched and grew to the L3 larval stage. 2-day-old worms were treated with the test compounds in 500 μl of S-medium with fresh HT115 RNAi bacteria culture, 1 mM IPTG and 25 μg ml^{-1} carbenicillin per well. The plates were incubated at 20°C for 24 hours and RNAi worms that had been treated with the same test compound were combined (24-wells). Worms were washed in M9 1X buffer and subjected to the measurement of Oil Red O staining intensity, or protein or RNA extraction.

Quantitative RT–PCR

Total RNA was extracted with Sepasol-RNA I Super G (Nacalai Tesque, Kyoto, Japan), purified with an RNeasy Mini Kit (Qiagen, Hilden, Germany), and reverse-transcribed into cDNA using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, U.S.A.) 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., Foster City, CA, U.S.A.) with IQ SYBR Green Super Mix (Bio-Rad, Hercules, CA, U.S.A.). 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: *ech-1* forward, 5'-GAGGCTAAGGCATTTGGTGA-3'; *ech-1* reverse, 5'-CGATTTCATTGACCGGAAGT-3';

acs-2 forward, 5'-TGACGTGCTCAAGTCTCCAC-3'; *acs-2* reverse, 5'-CTTCACCATCTTCTCGCACA-3';
cpt-5 forward, 5'-TGCGATGGAGCTGAGTTAGA-3'; *cpt-5* reverse, 5'-GTGACAGTCGCAATCTCCAA-3';
cdc-42 forward, 5'-AGCTTCATTCGAGAATGTCC-3'; *cdc-42* reverse, 5'-CTCGAGCATTCCTGGATCAT-3'.

Immunoblot Analysis

Two-day-old *C. elegans* were treated with 1 mM of various chemicals for 24 hours on NGM medium plated with *E. coli* OP50 as a food source. The nematodes were collected and washed with M9 1X buffer. After M9 buffer was removed, 50 μl of nematode sample was dispensed into Eppendorf tubes, protease inhibitor cocktail (Calbiochem, Billerica, MA, U.S.A.) was added, and 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 and, for each sample, 20 μg protein was loaded and separated by SDS-PAGE. Proteins were electro-transferred onto a polyvinylidene difluoride membrane (PVDF) (Millipore Immobilon-P, Billerica, MA, U.S.A.) and probed with phospho-Thr172-AMPK or phospho-Ser79-ACC [1/2000x dilution in 5% BSA/Tris buffered saline with Tween-20 (TBST)], followed by HRP-conjugated secondary donkey anti-rabbit IgG (1/5000x dilution in 5%BSA/TBST) and chemiluminescence was detected using ImageQuant LAS4010 (GE Healthcare Life Sciences, Buckinghamshire, U.K.). Blots were then stripped and reprobed with AMPK or ACC (1/2000x dilution in 5%BSA/TBST) and subjected to chemiluminescence detection.

Results and Discussion

Identification of alkaloids that affect lipid accumulation in worm using Nile Red and Oil Red O stain

C. elegans were treated with isoquinoline alkaloids such as berberine (protoberberine-type), sanguinarine (benzophenanthridine-type), magnoflorine

(aporphine-type), aromoline, tetrandine, and isotetrandine (bisbenzylisoquinoline-type) in the presence of Nile Red for 24 hours. 5-Aminoimidazole-4-carboxamide ribotide (AICAR) and alpha lipoic acid were selected as reference of lipid reducing agents (Fig. 0-1). AICAR, a nucleoside converted within cell to an AMP mimetic, is commonly used as AMPK activator which affects lipid metabolism resulting in lipid reduction (Fryer et al., 2002; Lee et al., 2006; Hawley et al., 2010). Alpha lipoic acid has known pharmacological effect to improve hypertriglyceridemia by stimulating triacylglycerol clearance and down-regulating liver triacylglycerol secretion.

After 24 hours, AICAR, berberine and sanguinarine treated worms showed marked reduction of lipid accumulation as reflected in weaker fluorescence intensity compared to untreated worms (Fig. 1-1A, B), whereas other alkaloids showed little or no reduction of fluorescence up to 5 mM concentration. At 1 mM concentration, alpha lipoic acid showed weak reduction of lipid. It is noted that with 24-hour treatment in this experiment set-up, no abnormality in the worms` growth or mobility associated with the lipid accumulation changes was observed.

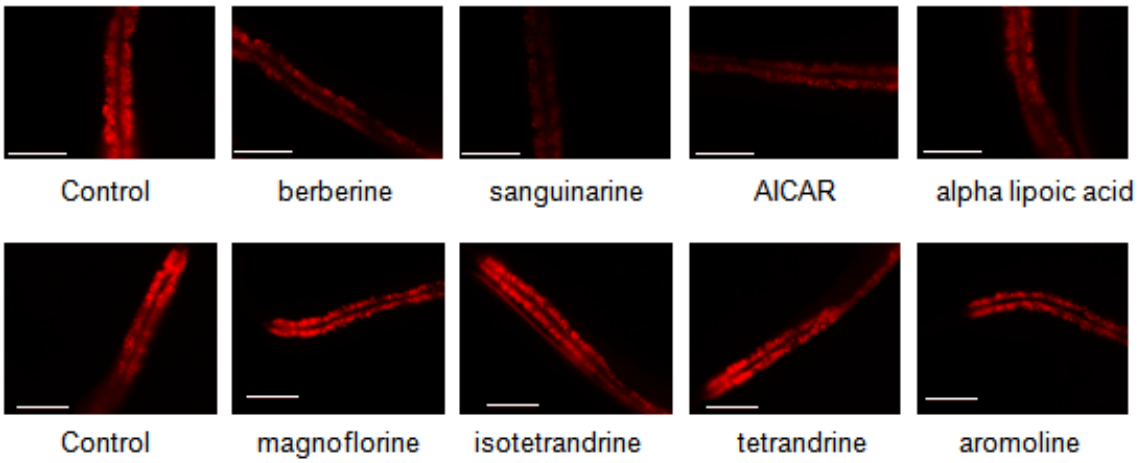


Fig. 1-1(A) Nile Red staining in treated worms. Berberine, sanguinarine, AICAR, and alpha lipoic acid were treated at 1 mM. Magnoflorine, isotetrandrine, tetrandrine, and aromoline were treated at 5 mM concentration. Images show typical results in worms treated with each test compound for 24 hours. Magnification is at 40× for top row and 20× for bottom row (Scale bar = 100 μm); exposure time = 1/25 s; light intensity = 40%.

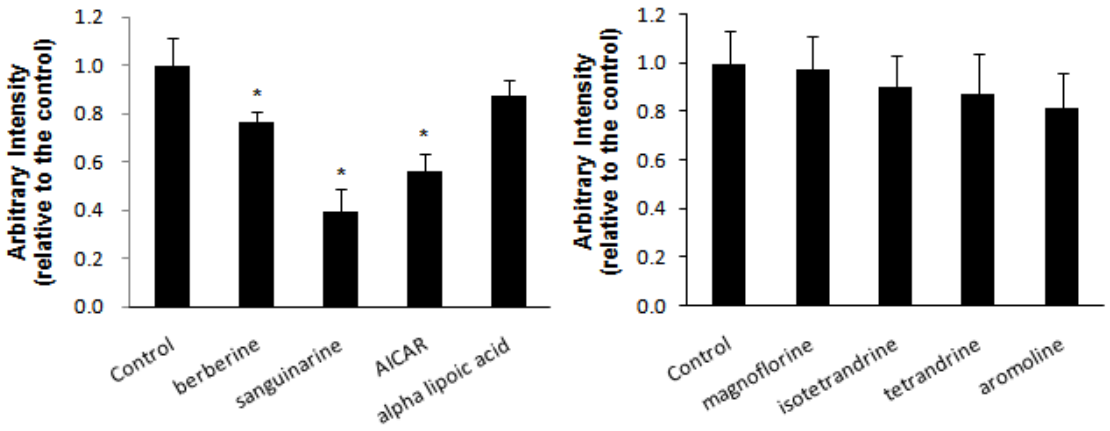
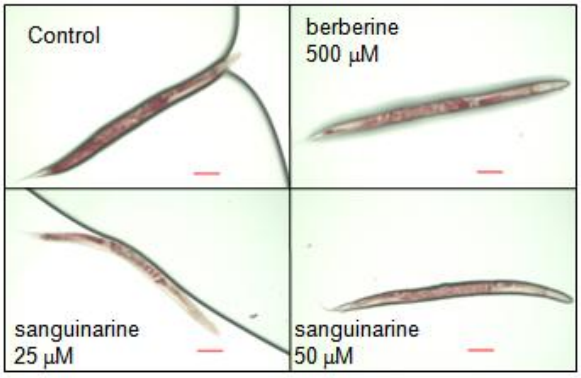


Fig. 1-1(B) Quantification of Nile Red fluorescence intensity in treated worms using ImageJ software; average values are shown for each compound tested and normalized to the control sample. n = 10 each from 3 independent experiments, error bars = SD. *$p < 0.05$ (ANOVA followed by Dunnett`s test).

The lipid reduction effects of berberine and sanguinarine were verified using Oil Red O stain (Fig. 1-1C, D). Among the non-effective compounds tested with Nile Red stain, only magnoflorine and tetrandrine were confirmed using Oil Red O stain due to limited availability of chemicals (Fig. 1-1 E, F).

(C)



(D)

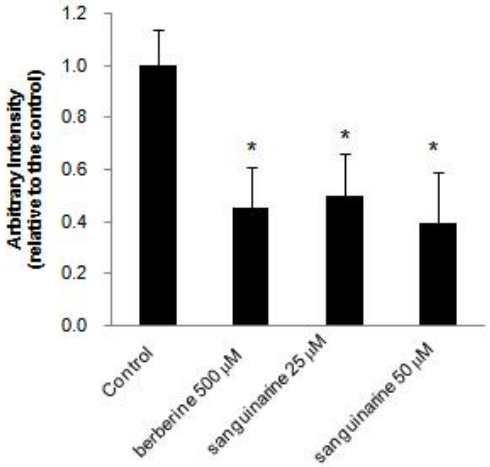
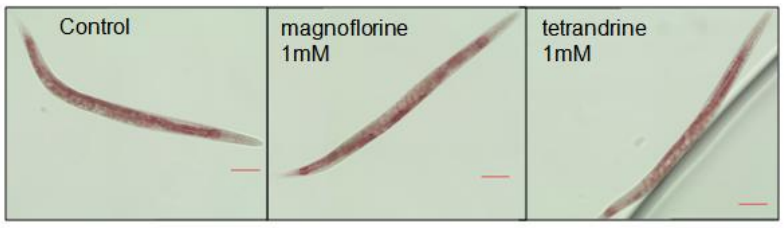


Fig. 1-1(C) Oil Red O staining in treated worms. Images show typical results in worms treated with berberine or sanguinarine for 24 hours. Magnification at 10× (Scale bar = 100 μm). (D) Quantification of Oil Red O staining intensity in treated worms using ImageJ; average values are shown for each compound tested and normalized to the control sample, error bars = SD. *$p < 0.05$ (ANOVA followed by Dunnett`s test).

(E)



(F)

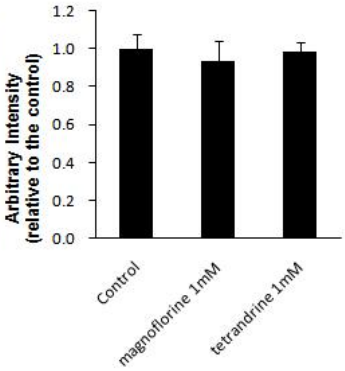


Fig. 1-1(E) Oil Red O staining in treated worms. Images show representative results in worms after treatment with magnoflorine or tetrandrine for 24 hours. Magnification at 20x (scale bar = 100 μm). n = 10-12 each from 3 independent experiments. (F) Quantification of the intensity of Oil Red O staining in treated worms using ImageJ. Values are the average of 10-12 images for each compound tested and are normalized to the control sample, error bars = SD.

The lipid-reducing effects of berberine and sanguinarine were found to be dose-dependent. Interestingly, sanguinarine exhibited more potent lipid reduction activity than berberine. When tested with lower concentration of sanguinarine, 25 μM still showed significant reduction of lipid accumulation, at almost 20-fold lower than the effective concentration of berberine (Fig. 1-2A, B). Further experiments were carried out to elucidate the difference in lipid reduction efficacy between berberine and sanguinarine based on the postulated pathways they act upon.

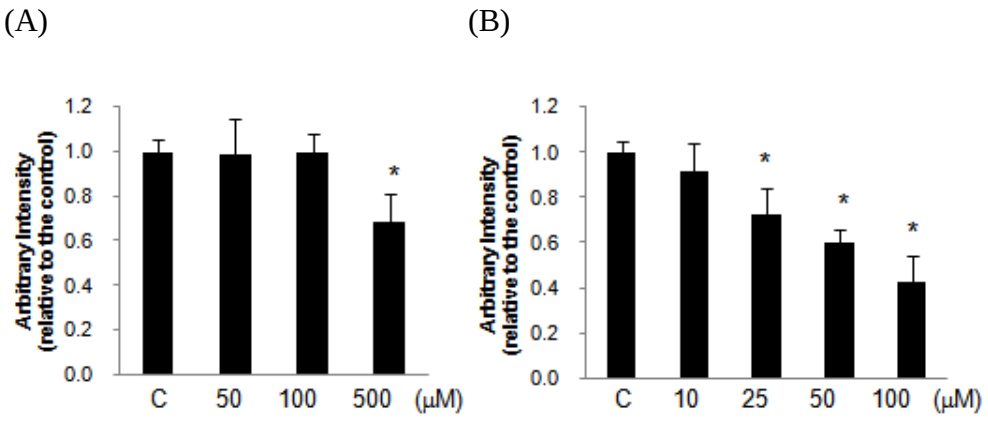


Fig. 1-2 Oil Red O staining in worms treated with (A) berberine and (B) sanguinarine for 24 hours. Magnification at 20x (scale bar = 100 μm). n = 10-12 each from 3 independent experiments, error bars = SD. *$p < 0.05$ (ANOVA followed by Dunnett`s test). C is control.

Enhanced phosphorylation of AMPK in berberine- and sanguinarine-treated worms

The involvement of AMPK pathway in berberine-induced fat reduction has been reported, whereby the inhibition of ACC in HepG2 cells resulted in the inhibition of cholesterol and triglyceride synthesis (Hawley et al., 2010; Brusq et al., 2006). Here, the effect of sanguinarine on AMPK and its downstream substrate, ACC, was further characterized in comparison with berberine. To examine the activation conditions of AMPK and ACC, their phosphorylation conditions were checked using anti-phospho AMPK (pAMPK)- and anti-phospho ACC (pACC)-specific antibodies. Sanguinarine and AICAR treatment for 24 h induced phosphorylation of AMPK but phosphorylation of ACC was insignificant. Meanwhile, berberine had only a marginal effect on AMPK

and ACC (Fig. 1-3A, B). Transient AMPK activation could be a possible reason for the weak phosphorylation detected. For instance, experiments using AICAR to activate AMPK had been reported whereby maximum phosphorylation activity was observed at 60 min (Pang et al., 2010) and at 2 h (Anilkumar et al., 2013) after which it gradually reduced to base level. Han et al. (2010) reported that berberine increased phosphorylation of AMPK and ACC in a dose- and time-dependent manner which peaked at 60 min after addition of 10 μM berberine to bovine aortic endothelial cells.

Since lipid reducing effect in worms using staining method was observed after 24 h alkaloid treatment, protein samples from 24 h-treated worms were used in earlier experiments. As follow-up, worms were treated with 500 μM berberine for 12 h and checked for AMPK and ACC activation conditions. Although statistically insignificant, increased AMPK phosphorylation was obvious when compared to 24 h treated worms (Fig. 1-3C, D). These results imply that there is a time lag between the peak of AMPK activation and the observable lipid-reducing effect. Therefore, time-course experiment would be necessary in future experiments to detect maximal AMPK activation by each test compound.

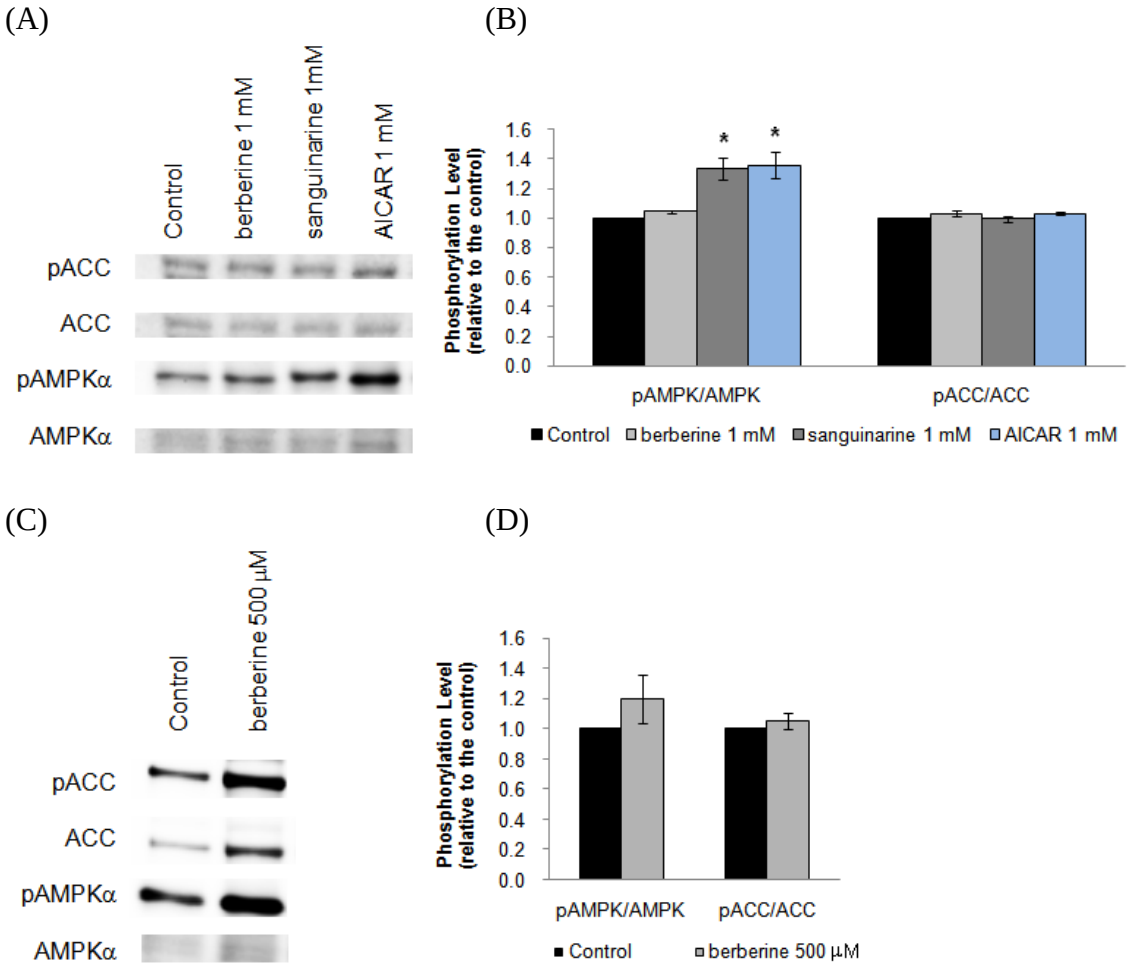


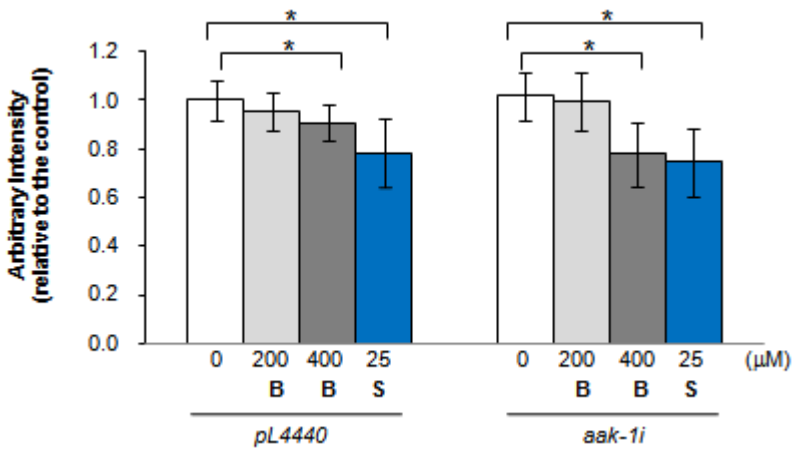
Fig. 1-3 Immunoblot showing phosphorylation of AMPK and ACC in treated worms. (A) Representative immunoblots of worm protein extracts for anti-phospho AMPKα (pAMPKα), anti-AMPKα (AMPKα), anti-phospho ACC (pACC) and anti-ACC (ACC) and (B) average phosphorylation levels of AMPK and ACC in worms treated for 24 h from three independent experiments. (C) Representative immunoblots and (D) average phosphorylation levels of AMPK and ACC in worms treated for 12 h from two independent experiments. Band intensities were quantified using ImageJ and normalized to total AMPK or ACC levels in control worms, error bars = SEM. *$p < 0.05$ (ANOVA followed by Dunnett's test).

Knockdown of the C. elegans homologs of catalytic α-subunits of AMPK revealed involvement of aak-2 in the lipid modulation mechanisms for berberine and sanguinarine

To investigate the mechanism through which AMPK activation might have induced lipid reduction in treated worms, the genes encoding the two *C. elegans* homologs of the catalytic α-subunits of AMPK, *aak-1* and *aak-2* were knocked down. AAK-1 and AAK-2 are 40% and 52% identical to, and they share 71% and 80% amino acid identity respectively with the kinase domain of the human AMPKα subunits, including conservation of a critical threonine residue whose phosphorylation is required for AMPK activation (Apfeld et al., 2004). The function of AAK-1 was much uncharacterized until a recent study by Lemieux et al. (2011) through which they identified an undisclosed compound F17 that activated AMPK signaling in *C. elegans* via *aak-1*.

Although phosphorylation of AMPK was observed, further RNAi experiments showed that lipid reduction activity of berberine and sanguinarine was still effective in *aak-1* but suppressed in *aak-2* RNAi worms (Fig. 1-4A, B).

(A)



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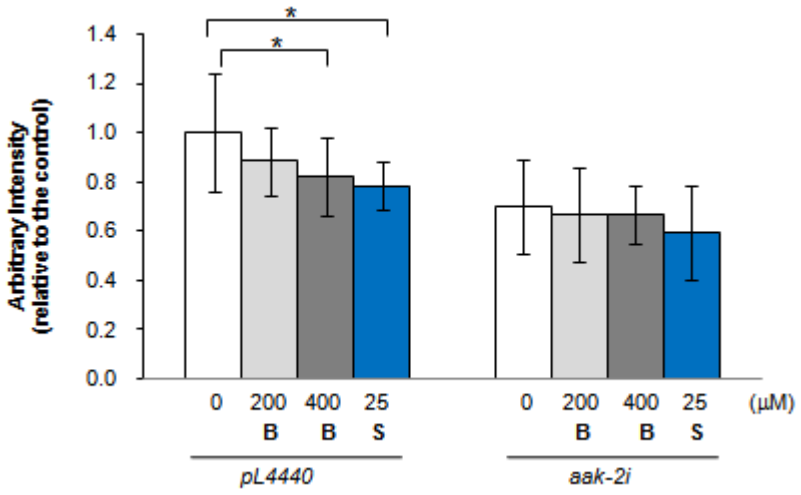


Fig. 1-4 Knockdown of the *C. elegans* homologs of catalytic α-subunits of AMPK revealed that *aak-2* was involved in the mechanisms of lipid-modulation for berberine and sanguinarine. The relative intensity of Oil Red O-stained lipid droplets quantified using ImageJ in (A) *aak-1* and (B) *aak-2* RNAi worms treated for 24 hours with berberine, **B** at 200 μM or 400 μM and sanguinarine, **S** at 25 μM. n = 10-12 for each compound tested; error bars = SD; *$p < 0.05$ (ANOVA followed by Dunnett`s test). *pL4440* – RNAi vector control, *aak-1i – aak-1 RNAi worms* , *aak-2i – aak-2* RNAi worms.

Although less viable compared to wild-type worms, *aak-1*(*tm1944*) mutants was reported to survive better than *aak-2*(*ok524*) mutants during larval L1 diapause (Fukuyama et al., 2012). It suggests that *aak-2* has a more critical function in promoting survival under nutritional stress condition. According to the findings of Li et al. (2006), the α1 and α2 isoforms of the catalytic AMPK subunits are present in most tissues but are differentially activated. Salt et al. (1998) reported that the α1-isoform was less activated than the α2-isoform in the rat's heart and skeletal muscle suggesting the lower sensitivity of the α1- than the α2-isoform complexes to smaller increases in AMP concentration.

In this study, quantitative real-time PCR analysis of the transcripts level of *aak-1* and *aak-2* in *aak-1* RNAi worms showed that *aak-2* expression was induced to a ratio comparable to the reduction of *aak-1* whereas the *aak-1* expression level was unaffected in *aak-2* RNAi worms (Fig. 1-5A, B). This result suggests that the worms' AMPK α-subunit expressions are regulated mainly through *aak-2* to maintain endogenous AMPK

level and it accounts for most of the basal activity and activation by berberine and sanguinarine. It also implies that, in this worm, the *aak-1* isoform of the AMPK α-subunit is less responsive to activation by berberine and sanguinarine.

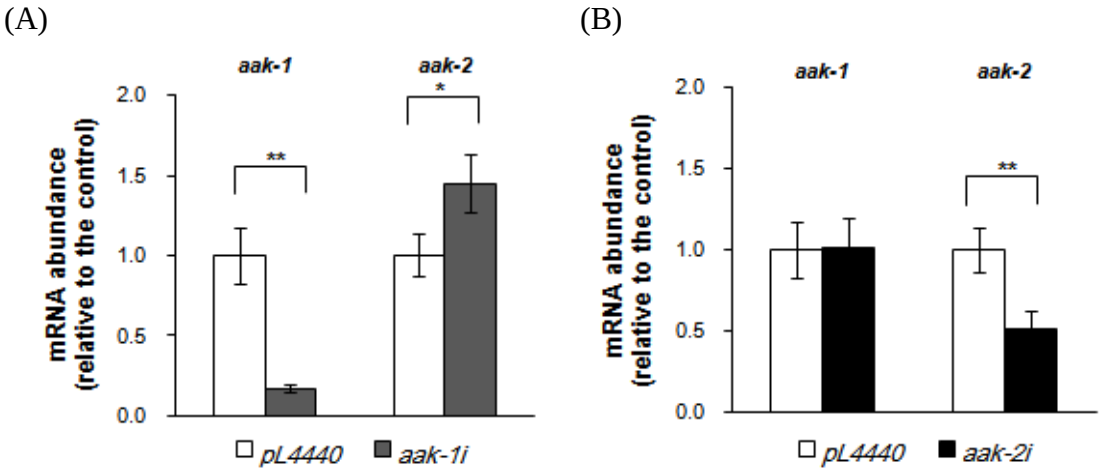


Fig. 1-5 Quantitative RT-PCR of *aak-1* and *aak-2* transcript levels in (A) *aak-1* and (B) *aak-2* RNAi worms. mRNA abundance values represent the average of triplicate cDNA templates applied to run qRT-PCR and the results were normalized against *cdc-42* as an internal control. Results were verified by reproducibility in at least two of three independent experiments and representative results from one experiment are shown, error bars = SD. *p < 0.05, **p < 0.005, two-tailed t-test. *pL4440* – RNAi vector control, *aak-1i – aak-1 RNAi worms, aak-2i – aak-2* RNAi worms.

Lipid- reducing effects of berberine and sanguinarine are independent of the nhr-49 regulated fatty-acid beta-oxidation pathway

Nuclear receptors (NRs) are a diverse class of transcription factors that mediate hormonal signaling processes in vertebrates and insects and now known to extend beyond direct transduction of endocrine signals to include responses to a variety of signaling molecules, 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, the remaining orphan NRs lack known cognate ligands (Gissendanner et al., 2004). 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 (Maglich et al., 2001), with a few of them exhibiting conserved physiological functions across taxa.

The *C. elegans* nuclear hormone receptor gene *nhr-49* has significant homology with hepatocyte nuclear factor 4 (HNF4) in mammals but its function is closer to the mammalian peroxisome proliferator-activated receptors (PPARs). *nhr-49* serves as a key regulator of fat usage by modulating fat consumption pathways and maintaining fatty acid saturation in balance. It targets multiple enzymes involved in transporting fatty acids across the mitochondrial membrane for β-oxidation. *nhr-49* knockout exhibited high-fat phenotype that was attributed to the deficiencies in two metabolic pathways, fatty acid β-oxidation and fatty acid desaturation. *nhr-49* was found to stimulate expression of a carnitine palmitoyl transferase (F09F3.9) which acts downstream of acyl-CoA synthetase in shuttling activated acyl-CoAs into the mitochondrial matrix (Van Gilst et al., 2005).

The involvement of β-oxidation genes targeted by NHR-49 in lipid reducing effects of berberine and sanguinarine was investigated through RNAi experiments. As mitochondrial β-oxidation process degrades stored fats for the production of energy, reduced expression of the key β-oxidation enzymes would result in high-fat storage. Consistent with the reported high-fat phenotype, *nhr-49* RNAi worms were found to be slightly larger in size and had increased fat storage with Oil Red O stain (Fig. 1-6A, B). They also had significantly lower expressions of mitochondrial β-oxidation genes, i.e., *ech-1* (encodes a mitochondrial β-oxidation trifunctional enzyme), *cpt-5* (encodes a carnitine palmitoyl transferase), and *acs-2* (encodes a mitochondrial acyl-CoA synthetase) (Fig. 1-7).

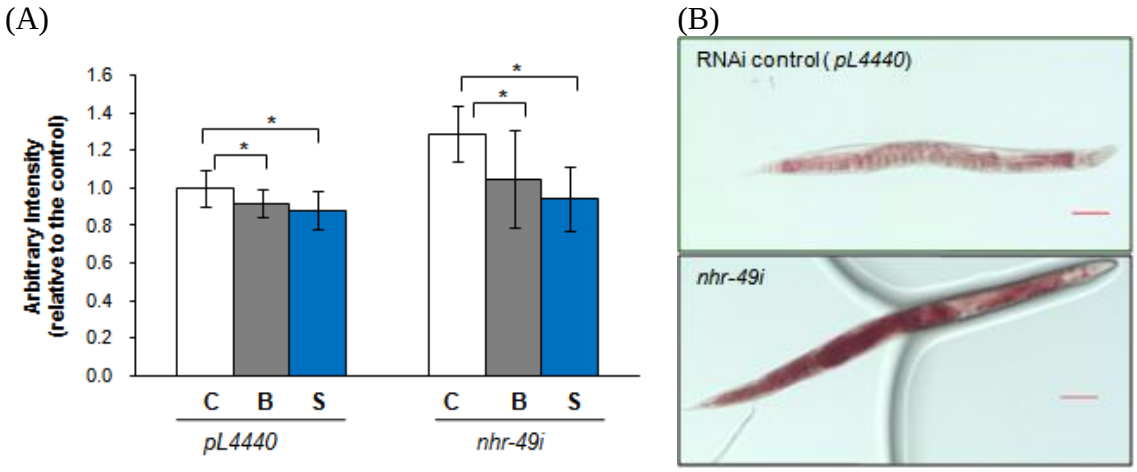


Fig. 1-6 Effects of alkaloid treatment on *nhr-49* RNAi worms. (A) Relative intensity of Oil Red O-stained lipid droplets quantified using ImageJ in *nhr-49* RNAi worms treated with berberine, **B** (400 μM) or sanguinarine, **S** (25 μM) for 24 hours. C is non-treated control; n = 10-12 for each compound tested, error bars = SD. **p* < 0.05 (ANOVA followed by Dunnett`s test). (B) Oil Red O staining of RNAi control worm and *nhr-49* RNAi worms. Images show typical results with worms at 20× magnification (Scale bar = 100 μm).

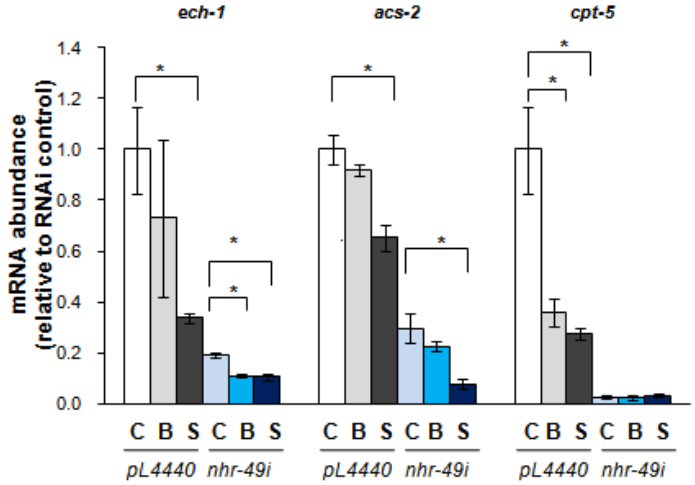


Fig. 1-7 Quantitative RT-PCR analysis on the expression of fatty-acid oxidation genes. The mRNA abundance value represents the average of triplicate cDNA template applied to run qRT-PCR and the results were normalized against *cdc-42*, error bars = SD. **p* < 0.05 (ANOVA followed by Dunnett`s test). *pL4440* – RNAi vector control, *nhr-49i* – *nhr-49* RNAi worm. Results were verified by reproducibility in at least two of three independent experiments.

Despite their high fat accumulation, *nhr-49* RNAi worms still retained sensitivity to berberine and sanguinarine (Fig. 1-6A), suggesting that both alkaloids act independently of *nhr-49* and their fat reducing activity would be on fat biosynthesis rather than its catabolism. However, analysis of *nhr-49* transcript level in RNAi control worms treated with berberine and sanguinarine showed reduced *nhr-49* expression (Fig. 1-8). These results suggest that they have antagonistic action on the lipid synthesis and degradation processes. While the details of the antagonistic action of berberine and sanguinarine on lipid synthesis (via AMPK) and degradation (via NHR-49) remains to be elucidated, the inhibition of lipid biosynthesis through AMPK activation showed greater effect on lipid accumulation.

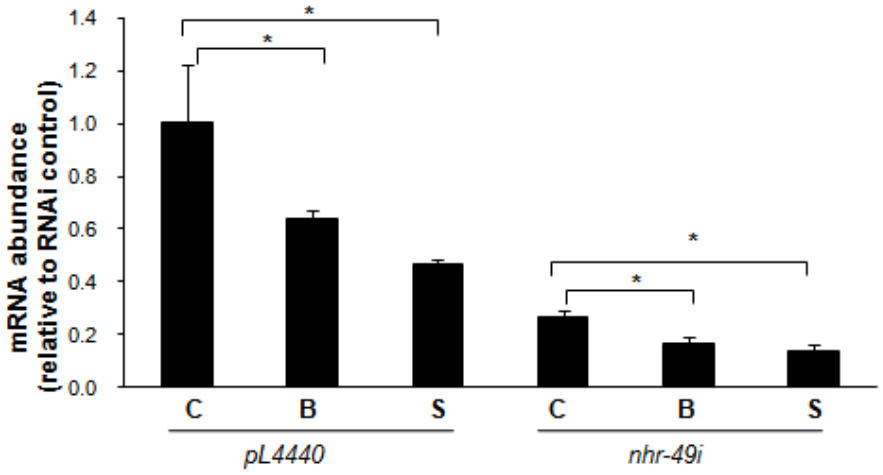


Fig. 1-8 Quantitative RT-PCR analysis on the expression of *nhr-49* gene. The mRNA abundance value represents the average of triplicate cDNA template applied to run qRT-PCR and the results were normalized against *cdc-42*, error bars = SD. *$p < 0.05$ (ANOVA followed by Dunnett`s test). *pL4440* – RNAi vector control, *nhr-49i* – *nhr-49* RNAi worm.

CHAPTER II

Application of the whole-worm model to screen for lipid reducing activities of metabolically-engineered plant cell cultures

Although many of the phytochemicals exhibit therapeutic effects, a lot of them still cannot be synthesized efficiently in the laboratory. One possible way to enhance production involves combination of biochemical knowledge, cloning, and transformation techniques, such as increasing the supply of bioactive compounds through metabolic engineering. The identification of many biosynthetic genes and characterization of the spatial and developmental regulation of their expression had proven their importance in the biosynthesis of secondary metabolites and revealed bottlenecks for their production in plant cells. Metabolic engineering of plant secondary metabolites biosynthesis pathway could improve quality of metabolites by reducing undesired pathways, introducing new pathways to produce novel compounds, or by completely blocking a pathway to accumulate intermediates (Chow and Sato, 2013). In the metabolic engineering of alkaloids, isoquinoline alkaloid biosynthesis has been most intensively investigated for production of bioactive compounds with pharmaceutical values.

Eschscholzia californica (California poppy) accumulates pharmacologically active alkaloids biosynthetically related to the morphinan alkaloids of *Papaver somniferum*. This, in combination with the relative ease with which it is propagated in vitro, makes it a key model for benzylisoquinoline biosynthesis (Loyola-Vargas and Vázquez-Flota, 2006). In this study, transgenic cell lines produced by heterologous expression of CYP80G2, a cytochrome P450 gene, from *Coptis japonica* in *E. californica* cells were screened for lipid-reducing effect in comparison with wild-type and vector control *E. californica* cell cultures. In addition, effect of *C. japonica* cell culture extract which contains main metabolite, berberine, was analyzed.

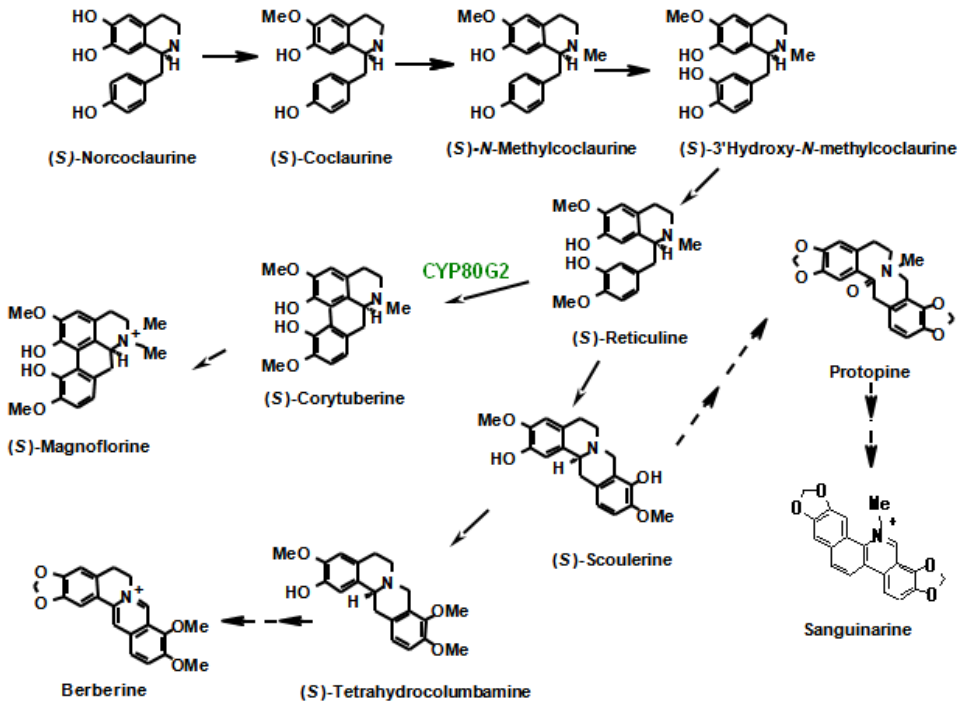


Fig. 2-0 Partial schematic of the isoquinoline biosynthesis pathway

Materials and Methods

Chemicals and reagents

Sanguinarine (Sigma), magnoflorine (a gift from R. Nishida, Kyoto University), and scoulerine (a gift from Mitsui Chemicals, Inc., Japan), berberine sulfate (Tokyo Chemical Industry Co., Ltd.), palmatine chloride (Mitsui Petrochemical Industries), coptisine chloride (Wako Pure Chemicals, Osaka, Japan), columbamine (prepared in our laboratory; Ikezawa et al.,2007). All other reagents were purchased from Wako Pure Chemicals unless otherwise stated.

Plant cell cultures

Wild-type, vector control and transgenic *CYP80G2*-overexpression *E. californica* cell lines were established and are maintained by our laboratory. The integration of the CYP80G2 expression construct in transgenic cells was confirmed by genomic PCR. Expression level of *CYP80G2* in those transgenic cells was further validated by qRT-PCR. The *C. japonica* (156-S) cell line was established by Sato et al. (2001) and is maintained in our laboratory.

LC-MS analysis of metabolites in E. californica cultured cells extracts

Constantly growing wild-type, vector control and transgenic *CYP80G2 overexpression* cells were used for the alkaloid analysis. Cultured cells were harvested after 14 d of culture and alkaloids were extracted from 2.0 g of fresh weight cells with 8 ml of methanol for 24 h. After homogenization and centrifugation, the alkaloids in supernatants were concentrated using rotary evaporator. The extracts were dissolved in distilled water and used for bioassay. The contents were determined by LC-MS 2010 (Shimadzu) with isocratic 40% (v/v) acetonitrile/H_2O solvent containing 0.05% (v/v) trifluoroacetic acid (TFA) at constant flow rate of 0.8 ml/min. Column = TOSOH TSK-Gel ODS 80-Tm silica-based, reversed phase 4.6 x 250 mm; column temperature = 40°C. UV absorbance was measured at 280 nm; mass range (m/z 100 to 800) by both single-ion and scan modes. Sanguinarine, magnoflorine, and scoulerine were identified by direct comparison with standard chemicals in LC-MS analysis. Identification of other main peaks was deduced from their m/z values based on previous study (Takemura et al., 2010). Alkaloids concentrations were quantified relative to the peak area of standards.

LC-MS analysis of metabolites in C. japonica cultured cells extracts

10.0 g of fresh weight *C. japonica* (156-S) cells were soaked in 100 ml methanol for 48 h. The filtered extract was concentrated using rotary evaporator and dissolved in distilled water for bioassay and analysis by LC-MS 2010 (Shimadzu). H_2O(A)/acetonitrile (B) solvent containing 0.05% (v/v) TFA was used at constant flow rate of 0.5 ml/min. After initial hold at 35% B for 15 min, linear gradient was applied from 35% to 70% B in 4 min, hold at 70% B for 3 min, 70% to 35% B in 4 min and finally hold at 35% B for 4 min. Column type, column temperature and detection parameters were same as above. Peaks were identified by direct comparison with standard chemicals and alkaloids concentrations were quantified relative to the peak area of standards.

C. elegans bioassay

Two-day-old worms were treated with distilled water (as control), cell extracts, or alkaloids for 24 hours. About 200-300 worms were collected and washed three times

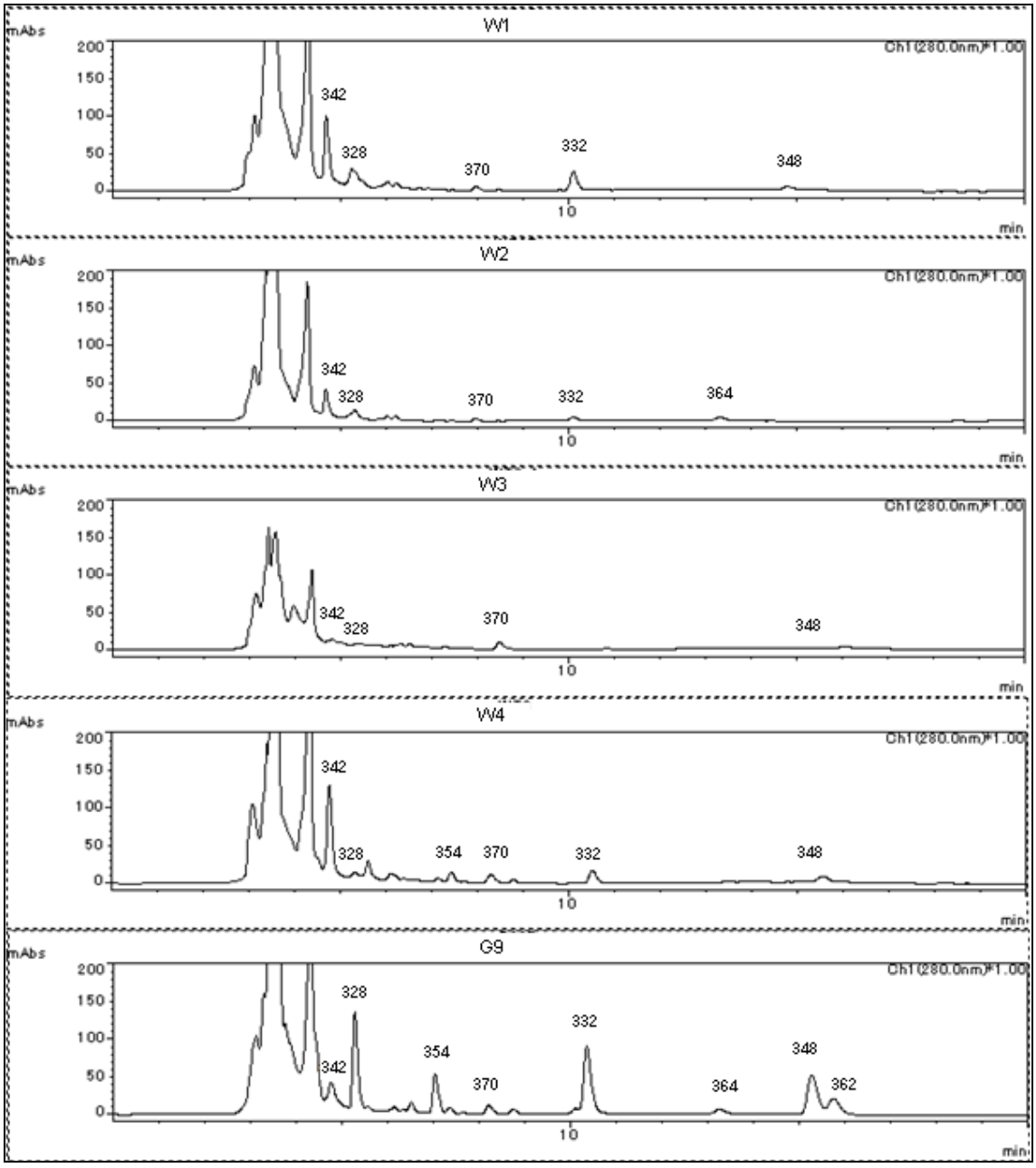
with 1X PBS pH 7.4 buffer. Oil Red O staining and its quantification were performed as described in Chapter I.

Results and Discussion

Alkaloid profiles of E. californica cultured cells extracts

Since CYP80G2 has been identified as corytuberine synthase (Ikezawa et al., 2008) acting on substrate, (*S*)-reticuline, this infers that heterologous expression of *CYP80G2* gene from *C. japonica* in *E. californica* cells may alter its alkaloid biosynthesis pathway to branch off at (*S*)-reticuline and produce (*S*)-corytuberine and/or magnoflorine as intermediate or end-products. It could also concurrently assume its inherent pathway to produce (*S*)-scoulerine, sanguinarine, or accumulate other intermediates (Fig. 2-0).

LC-MS analysis of the cell extracts (Fig. 2-1) revealed similar alkaloids composition in wild-type (W), vector control (G) and *CYP80G2*-overexpression (C) culture cells. The main peaks identified based on known m/z values are magnoflorine (m/z 342), scoulerine (m/z 328), sanguinarine (m/z 332), protopine (m/z 354), allocryptopine (m/z 370), 10-hydroxychelerythrine (m/z 364), chelerythrine (m/z 348), chelirubine (m/z 362). The *CYP80G2*-overexpression culture cell lines have similar or higher magnoflorine content compared to the wild-type or vector control lines. However, accumulation of scoulerine and sanguinarine were also found. It is noted that line C9 had remarkably higher alkaloid content compared to other transgenic cell lines with the same mass (Fig. 2-2).


WI1
mAbs
Ch1(280.0nm)*1.00
342
328
370
332
348
min
WI2
mAbs
Ch1(280.0nm)*1.00
342
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332
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WI3
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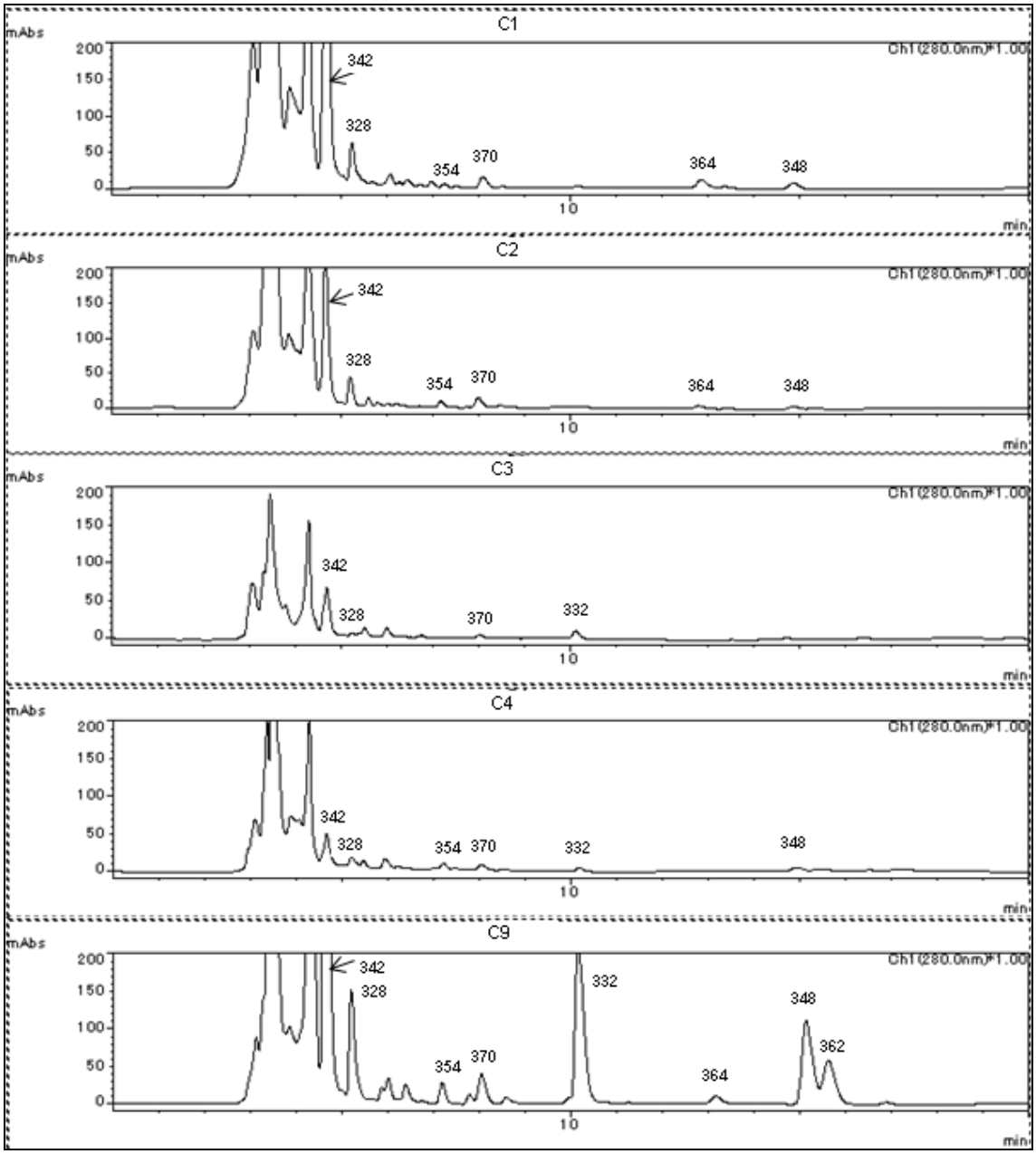


Fig. 2-1 Liquid chromatogram of wild-type (W), vector control (G) and CYP80G2-overexpression (C) *E. californica* cell extracts. Labeled peaks indicate m/z 342 (magnoflorine), m/z 328 (scoulerine), m/z 332 (sanguinarine), m/z 354 (protopine), m/z 370 (allocryptopine), m/z 364 (10-hydroxychelerythrine), m/z 348 (chelerythrine), m/z 362 (chelirubine).

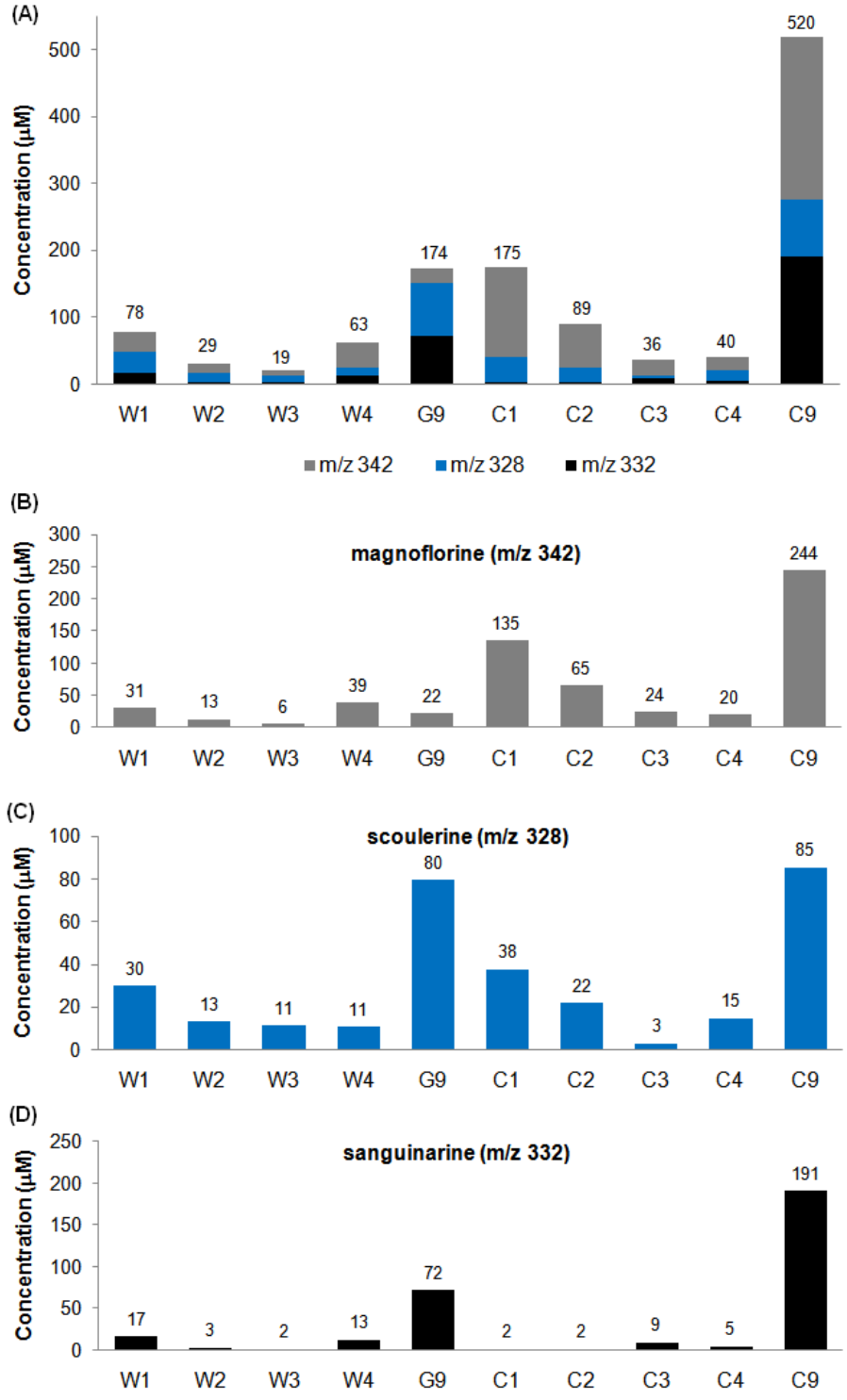


Fig. 2-2 (A) Total concentration of three alkaloids and the respective concentration for (B) magnoflorine (m/z 342), (C) scoulerine (m/z 328) and (D) sanguinarine (m/z 332) in *E. californica* cell extracts. Semi-quantitative values are presented here as µM equivalence of the respective alkaloid calculated relative to the peak area of each standard.

Lipid-reducing effect of E. californica cell extracts in C. elegans

Extracts from wild-type (W), vector control (G) and CYP80G2-overexpression (C) culture cells with the alkaloids profile and concentration shown in Fig. 2-1 and 2-2 were fed to the worms for 24 h and effects on lipid accumulation was observed using Oil Red O stain. Among the extracts from 10 culture cell lines tested, only C9 showed significant lipid reduction in worms (Fig. 2-3). One obvious factor for the effectiveness of C9 is its high alkaloids content (244 μM magnoflorine, 85 μM scoulerine and 191 μM sanguinarine) compared to other cell line extracts (Fig. 2-2A). By comparison of the alkaloids composition of various cell line extracts, the profile of G9 resembles that of C9 with similar concentration of scoulerine (80 μM) and high sanguinarine(72 μM) content. Although G9 contained only 22 μM magnoflorine, it is known from earlier results that magnoflorine did not have lipid-reducing effect in the worms (Chapter I, Fig. 1-1F). This ruled out the contribution of magnoflorine in the lipid-reducing effect in treated worms. Since both lines contained similar amount of scoulerine at 85 μM (C9) and 80 μM (G9) but G9 extract did not have significant lipid-reducing effect in treated worms, it showed that scoulerine was ineffective at lipid-reduction at those concentrations. Moreover, C1 extract, with relatively high content of magnoflorine (135 μM) and scoulerine (38 μM) but low content of sanguinarine (2 μM) as compared to C9, did not reduce lipid accumulation in the worms. These results suggest that sanguinarine is the main contributor to the lipid-reduction activity observed with C9 extract.

Although the effective concentration of sanguinarine was not determined here, it was noted that its lipid-reducing effect was weaker in the presence of other metabolites contained in plant cell culture extracts. For example, G9 which contained 72 μM sanguinarine did not yield lipid-reducing effect whereas 25 μM sanguinarine was sufficient to yield such effect when applied alone (Fig. 1-1D).

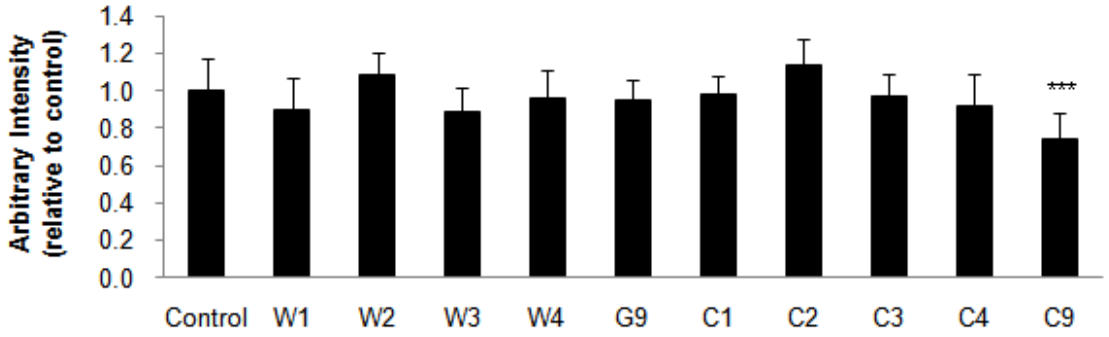


Fig. 2-3 Quantification of Oil Red O staining intensity in treated worms using ImageJ; average values are shown for10-12 images of each extract tested and normalized to the control sample, error bars = SD. ***$p < 0.001$ (ANOVA followed by Dunnett`s test).

Lipid-reducing effect of C. japonica cell extracts in C. elegans

The lipid-reducing effect of berberine was confirmed using the *C. elegans* screening model although its activity was lower than sanguinarine (Chapter I). Several medicinal plants such as *Coptis* contain berberine. Thus, I examined the effect of the extracts from high berberine-producing 156S cells of *C. japonica* on lipid reduction of the worm. LC-MS analysis indicated that *C. japonica* 156S cell extract contained berberine (m/z 336), magnoflorine (m/z 342), coptisine (m/z 320), palmatine (m/z 352), and some unidentified metabolites with m/z 324 and m/z 338 (Fig. 2-4).

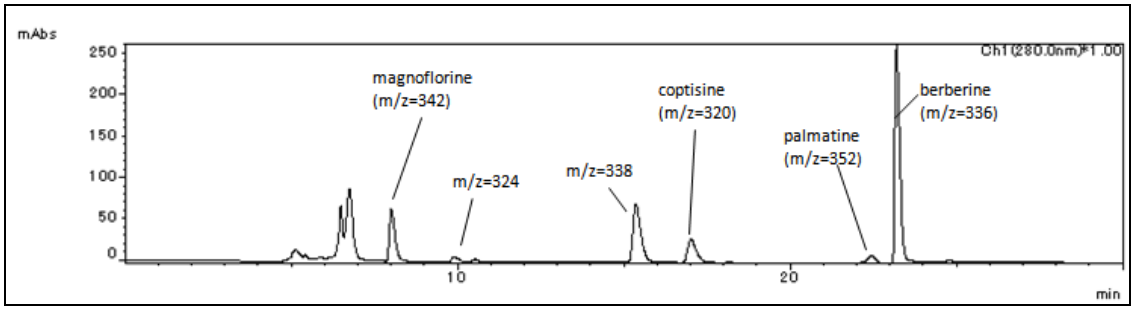


Fig. 2-4 Liquid chromatogram of *C. japonica* (156S) cell extract.

The *C. japonica* 156S cell extract was diluted to a final concentration of about 109 μM berberine, 5 μM palmatine and 23 μM coptisine and applied to the worms as in Chapter I. Oil Red O staining of worms after 24 h treatment showed that *C. japonica* cell culture extract had stronger lipid-reducing effect than 100 μM berberine applied alone (Fig. 2-5).

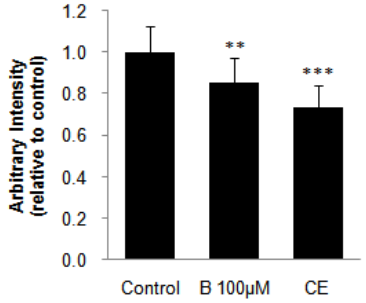


Fig. 2-5 Quantification of Oil Red O staining intensity in treated worms using ImageJ; average values are shown for10-12 images of each treatment and normalized to the control sample, error bars = SD. $**p < 0.01$, $***p < 0.001$, two-tailed t-test. B – berberine, CE – cell extract.

Mixed compositions in extracts of E. californica and in C. japonica cell cultures affect lipid-reducing activity differently

Plant extracts with a mixed composition of secondary metabolites are commonly applied as traditional therapeutics. The combination of active components is believed to produce synergistic effect for treatment of different ailment. The results here showed that the *C. japonica* cell extract have a stronger lipid-reducing effect than berberine alone. This implies a synergistic effect between berberine and other components in the extract. In fact, some of the protoberberine alkaloids showed similar effects on lipid accumulation as berberine (Kawasaki, unpublished results). On the contrary, results from treatment with *E. californica* cell extract suggest other components had an antagonistic effect on sanguinarine which is more effective when applied alone.

Besides the major alkaloids identified here by LC-MS analysis, there are other bioactive components present in the cell extracts including phenolic compounds such as flavonoids, polyphenols and phenolic acids yet to be analyzed. Phenolic compounds are reported to have antioxidant, antimicrobial, and antitumor properties (Guimarães et al.,

2013; Wang et al., 2013; Dias et al., 2013) and their presence in crude extracts could affect the overall bioactivity. In this study, the results indicate that the interactions between protoberberine-type alkaloid and other metabolites in the *C. japonica* cell extracts yield different bioactivity strength than that of the benzophenanthridine-type alkaloids found in *E. californica* cell extract. Results of C9 suggests that its bioactivity is more related to the constituents profile than to the amounts present in the extract since the effective concentration of sanguinarine was determined earlier to be 25 μM.

It is interesting that the non-alkaloid components might be synergistic in one extract and antagonistic in another. While synergism of alkaloids with other plant secondary metabolites had been reported (Eid et al., 2012a), antagonistic effects were found in other studies. Dias et al. (2013) reported that purified extracts of wild fruits, *Arbutus unedo*, *Prunus spinosa*, *Rosa micrantha* and *Rosa canina*, exhibited higher antioxidant properties than the crude extracts in which antagonistic effects between the compounds present resulted in a decrease in the antioxidant activity. While further investigations are needed to identify and compare the other non-alkaloid chemical constituents between *C. japonica* and *E. californica* cell cultures and to evaluate their effects on the overall bioactivity of extracts, this worm model is useful in the preliminary screening as it reflects a more comprehensive response of an organism as a whole compared to in-vitro or cell-based assays. Such studies will help elucidate the interaction of bioactive chemical constituents in cell extracts for therapeutical applications in the future.

CHAPTER III

RNAi knockdown of *nhr-8* enhanced worms' sensitivity to bioassay

As described in previous chapters, *C. elegans* is a good model organism for isoquinoline alkaloids bioassay. However, this worm is less sensitive to chemicals compared to other assay systems such as mammalian cell cultures (Broeks et al., 1995; Kong et al., 2004; Burns et al., 2010). Under natural conditions, *C. elegans* are exposed to large range of chemical assaults found in soil. Besides feeding on microbes, it behaves like a scavenger in the soil environment, consuming compounds from other animals, microorganisms, plants, and xenobiotics. As a result, it requires efficient detoxification ability of organic chemicals for survival. Recent studies have identified many genes encoding sensors and enzymes that the worms may use in their xenobiotics responses such as a large number of four main classes of detoxification enzymes including cytochromes P450 (CYP), short-chain dehydrogenases (SDR), UDP-glucuronosyl or glycosyl transferases (UGT), and glutathione-S-transferases (GST) (Lindblom and Dodd, 2006).

Most studies on *C. elegans* detoxification mechanism were attempted to circumvent its xenobiotic resistance in screening for more effective nematicide candidates among compounds that exhibit toxic property, including plant secondary metabolites. *nhr-8* is a gene which encodes a nuclear hormone receptor involved in xenobiotic resistance response of the *C. elegans*. Although the actual target genes of *nhr-8* have not been identified yet, the precedent PXR (pregnane X receptor) and CAR (constitutive androstane receptor) activities in vertebrates suggested that NHR-8 may regulate the expression of the cytochrome P450 genes in *C. elegans*. The toxin sensitivity of *nhr-8* is specific as *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* (Lindblom et al., 2001).

The function of plant alkaloids has been suggested as defense against herbivores and pathogens, therefore they are likely to induce xenobiotic response and consequently their bioavailability might be reduced in vivo. Accordingly, the isoquinoline alkaloids, berberine and sanguinarine, were also expected to induce xenobiotics response in the

worms. The genome-wide response of *C. elegans* to berberine and sanguinarine treatment was previously investigated by 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 worm had been used to investigate its sensitivity to toxic compounds that act as nematicide, there is no report on studies done to explore *nhr-8* RNAi worms' response to the bioactivities of the compounds being treated with. 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 the *nhr-8* RNAi worms with lower alkaloid concentration to check if this could increase the alkaloid bioavailability in those worms. Next, alkaloids accumulation in the worms was analyzed to investigate if the increased sensitivity to alkaloid treatment could be due to higher bioavailability in the RNAi worms. In addition, the differences in detoxification genes induction between the alkaloid-treated RNAi control and *nhr-8* RNAi worms were also analyzed.

Materials and Methods

Chemicals and reagents

As described in Chapter I.

Nematode treatment

As described in Chapter I.

Oil Red O staining

As described in Chapter I.

RNA interference of nhr-8

An RNAi feeding method (Lehner et al., 2006) was used as described in Chapter I. Part of the nucleotides of the coding regions of *nhr-8* complementary DNA was 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 were used to amplify the genes of interest. Each cDNA segment was cloned into feeding vector pL4440 (A. Fire, Stanford University) with respective restriction sites and transformed into HT115 bacterial cells.

Quantitative RT–PCR

To measure the expression levels of xenobiotic response genes, qRT-PCR was done as described in Chapter I. The relative amount of transcript between samples was 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'-AATTGGAGGACATCCTGTCG-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'-TGCAAGCATATTCGCATTTC-3';

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

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

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

Worms were washed with 0.1% SDS solution and then with 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 μg/ml proteinase K) with coptisine chloride added as internal standard. The lysis samples were incubated 60°C for 1 h and then kept on ice. 100 μl cold acetonitrile was added to each lysate,

mixed by pipeting, and centrifuged at 800 x *g* for 5 min. 40 μl of sample was injected for analysis by LCMS2010 system (Shimadzu) with the following parameters:
Column = Cosmosil 4.6 x 250 mm $5C_{18}$-AR-300; Column temperature = 27°C;
Solvent A = 4.9:95:0.1 (acetonitrile/H_2O/TFA);
Solvent B = 95:4.9:0.1 (acetonitrile/H_2O/TFA) using gradient elution as shown below.

Time (min)	Solvent	Flow rate (ml/min)
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) by both single-ion and scan modes.

Accumulation of alkaloid and metabolites were calculated based on LC peak area relative to the internal standard peak of coptisine. This value is normalized to 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.

Results and Discussion

RNAi knockdown of nhr-8 enhanced lipid reduction activity of berberine

In previous experiments with wild-type N2 worms (Chapter I), treatment with 500 μM berberine and 25 μM sanguinarine consistently yielded detectable lipid reduction activity through Oil Red O staining, but lower concentration such as 50 μM berberine and 10 μM sanguinarine were ineffective. In this study, the worms' detoxification process was impeded by knocking down *nhr-8* and the effects of alkaloid treatment on those worms were examined. Results of *nhr-8* RNAi worms showed that 50 μM

berberine was effective for lipid reduction at statistically significant level ($p < 0.005$) after 24 hour treatment (Fig. 3-1A), whereas this concentration was ineffective in wild-type worms. Although lipid reduction effect was also observed at 10 μM sanguinarine in RNAi control worms, the difference between *nhr-8* and the control RNAi worms treated with sanguinarine was significant ($p < 0.05$) (Fig. 3-1B).

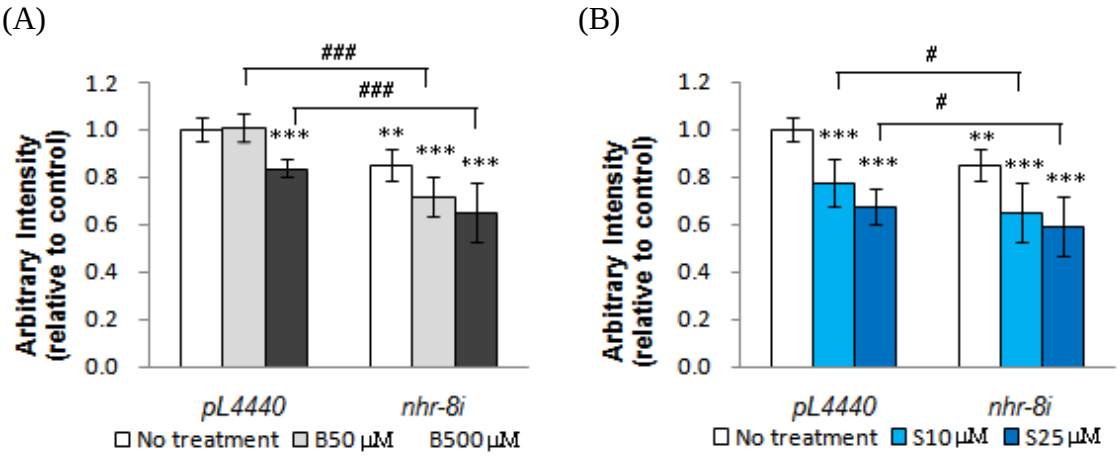


Fig. 3-1 Effects of (A) berberine and (B) sanguinarine on lipid accumulation in RNAi control worm and *nhr-8* RNAi worms. Quantification of Oil Red O staining intensity using ImageJ. Values are the averages of 10-12 images for each compound tested and normalized to the RNAi control sample; error bar = SD; $^{**}p < 0.005$, $^{***}p < 0.001$; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ (ANOVA followed by Dunnett`s test). B – berberine, S – sanguinarine.

In this experiment, I used only berberine (protoberberine-) and sanguinarine (benzophenanthridine)-type alkaloids to evaluate the susceptibility of *nhr-8* RNAi worms to the alkaloids' lipid-reducing effect. The results showed berberine`s effect was more enhanced than sanguinarine's effect on the *nhr-8* RNAi worms. This enhanced sensitivity to berberine suggests that detoxification process was induced in the worms and may have affected their sensitivity to chemical treatment. Therefore, *nhr-8* RNAi worms would be useful for future screening experiments to evaluate the full activity of test compounds and their sensitivity to detoxification. Moreover, the amount of complex plant alkaloids isolated for bioassay could also be reduced.

Accumulation of alkaloids in nhr-8 RNAi worms at low dosage

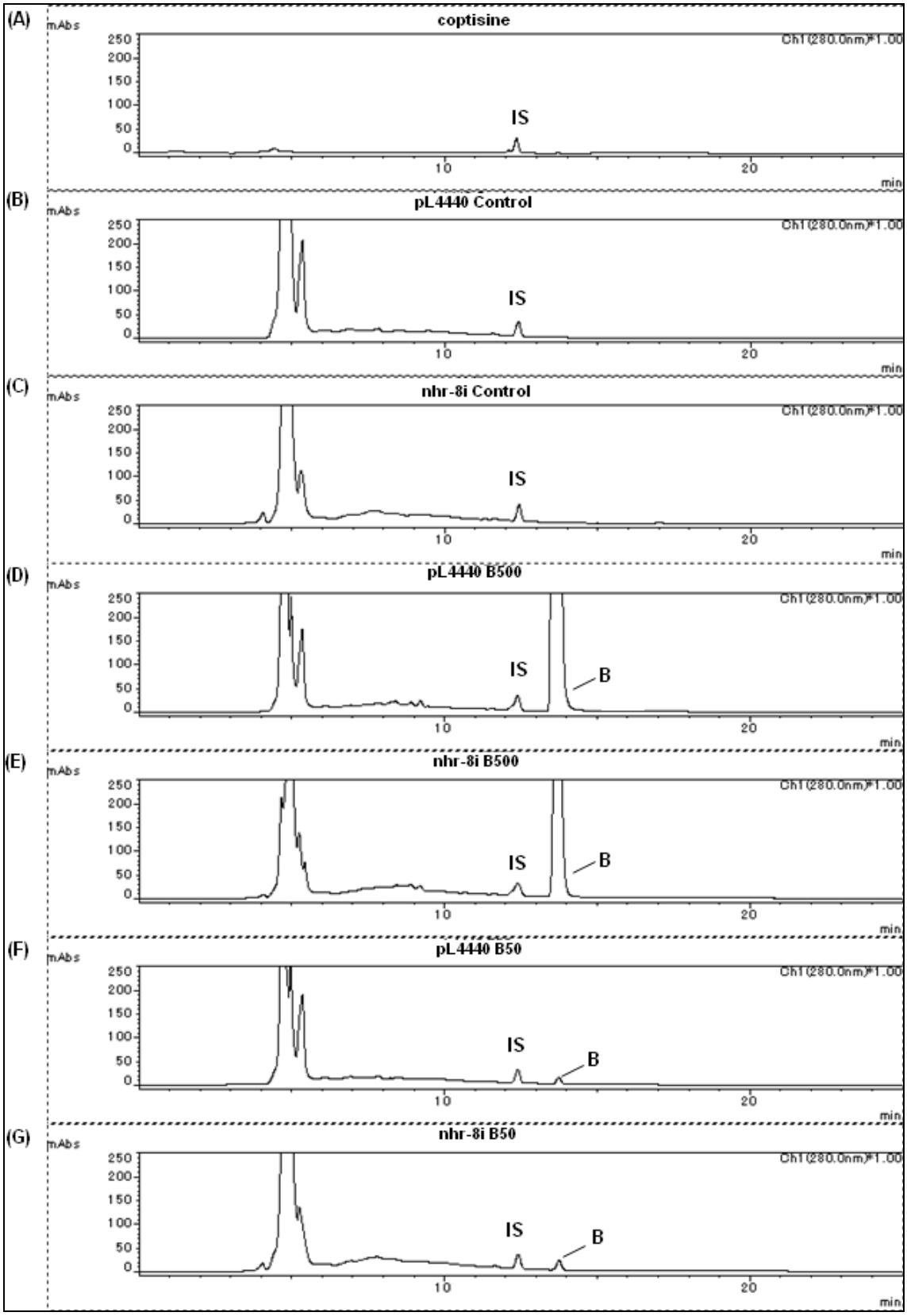
Next, to investigate the enhanced sensitivity of *nhr-8* RNAi worms to berberine and sanguinarine treatment, the accumulation of alkaloids and their metabolites in the worms was analyzed to examine whether the bioavailability of alkaloids was modified by *nhr-8* knockdown.

In berberine-treated worm samples, four major metabolites of berberine were monitored based on the metabolic analyses reported for mammalian cells, i.e., berberrubine (m/z 322), thalifendine (m/z 322), demethyleneberberine (m/z 324) and jatrorhizine (m/z 338) in rat plasma, human liver microsomes, and in rat and human urine (Zuo et al., 2006; Qiu et al., 2008; Li et al., 2011; Ma et al., 2013).

On the other hand, study on the metabolism of sanguinarine is rather limited. Besides dihydrosanguinarine (m/z 334), which was reported as the main metabolite of sanguinarine, some metabolites such as m/z 334 after ring cleavage, m/z 320 after successive O-demethylation or m/z 336 after ring-cleavage of m/z 334 were also detected in human, rat and pig liver microsomes (Deroussent et al., 2010; Zhang et al., 2013).

Using these metabolic analyses data for berberine and sanguinarine, the alkaloid metabolites in treated worms were analyzed based on the reported m/z values using LC-MS (Fig. 3-2, 3-3), since standards of those metabolites were unavailable. Preliminary experiments using wild-type worms in 6 h and 24 h treatment showed no distinct peak difference in metabolite profiles between 6 h and 24 h. The culture media samples also showed no difference between 6 h and 24 h. Therefore, in *nhr-8* RNAi experiment, 24 h worm samples were analyzed.

In berberine treatment, only accumulation of berberine was detected and no molecular ion peak that corresponds to the 4 major metabolites (berberrubine, thalifendine, demethyleneberberine and jatrorhizine) nor the glucuronide conjugates was found. Thus, the change in berberine content was analyzed in *nhr-8* RNAi experiment. On the other hand, in sanguinarine treatment, accumulation of sanguinarine in the worms was low and two metabolites of sanguinarine (m/z 334, m/z 336) were detected. Thus, these metabolites contents were analyzed.


(A)
coptisine
mAbs
Ch1(280.0nm)#1.00
IS
min
(B)
pL4440 Control
IS
(C)
nhr-8i Control
IS
(D)
pL4440 B500
IS
B
(E)
nhr-8i B500
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B
(F)
pL4440 B50
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(G)
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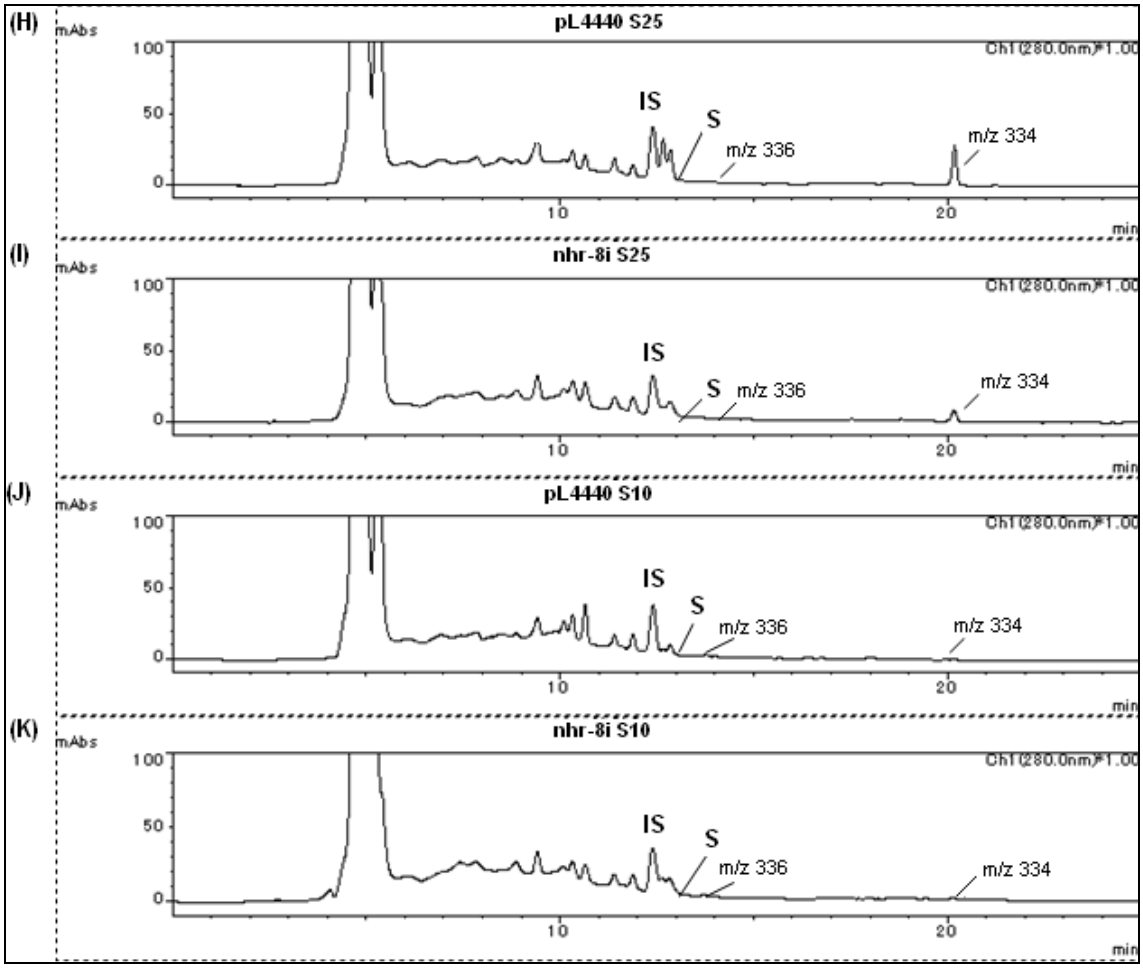

Fig. 3-2 Liquid chromatogram of (A) coptisine (IS), and alkaloids extracted from worms, i.e., (B) *pL4440* control, (C) *nhr-8i* control, (D) *pL4440* B500 μM, (E) *nhr-8i* B500 μM, (F) *pL4440* B50 μM, (G) *nhr-8i* B50 μM, (H) *pL4440* S25 μM, (I) *nhr-8i* S25 μM, (J) *pL4440* S10 μM, (K) *nhr-8i* S10 μM.
pL4440 – RNAi vector control, *nhr-8i* – *nhr-8* RNAi worm. IS – internal standard, B – berberine (m/z 336), S – sanguinarine (m/z 332).

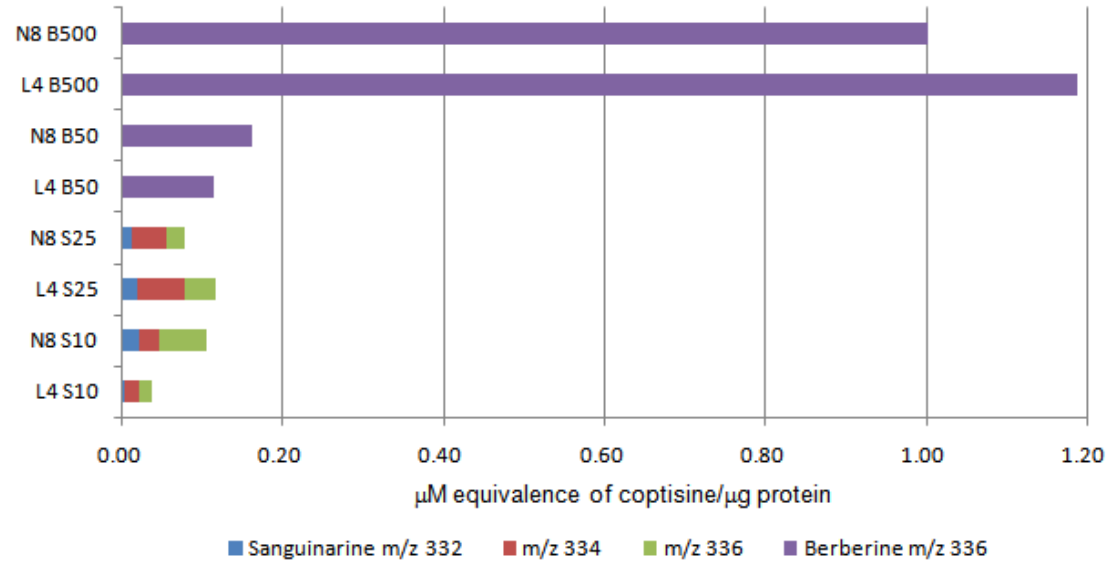

Fig. 3-3 Accumulation of berberine, sanguinarine and their metabolites in RNAi vector control and *nhr-8* RNAi worms. Semi-quantitative values are presented here as µM quivalence of coptisine, (internal standard used in worm extraction process). These values represent the one of three independent experiments. 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.

Although I expected that the *nhr-8* RNAi worms would have accumulated more alkaloids when the detoxification response was knocked down, such increases in alkaloid contents were only detected at low dosages, i.e. 50 µM berberine and 10 µM sanguinarine but not at high dosages, i.e. 500 µM berberine (Fig. 3-4A) and 25 µM sanguinarine (Fig. 3-4B). Since the availability of alkaloids in vivo would be critical for biological activity, the increase in alkaloids accumulation in *nhr-8* RNAi worms at low concentration is an important finding. The genetic analyses of cytoprotective mechanisms in *C. elegans* suggest that such mechanisms may function efficiently at low doses but are overwhelmed at high doses (Shore and Ruvkun, 2013). This correlates with higher alkaloid retention in the worms at higher dosages in current results (Fig. 3-4A, B). However, it is unclear why *nhr-8* RNAi worms accumulated less alkaloid than RNAi control worms at high dosage. As discuss later, there are other orphan receptor genes involved in xenobiotic metabolism and *nhr-8* knockdown might enhance the other detoxification pathways.

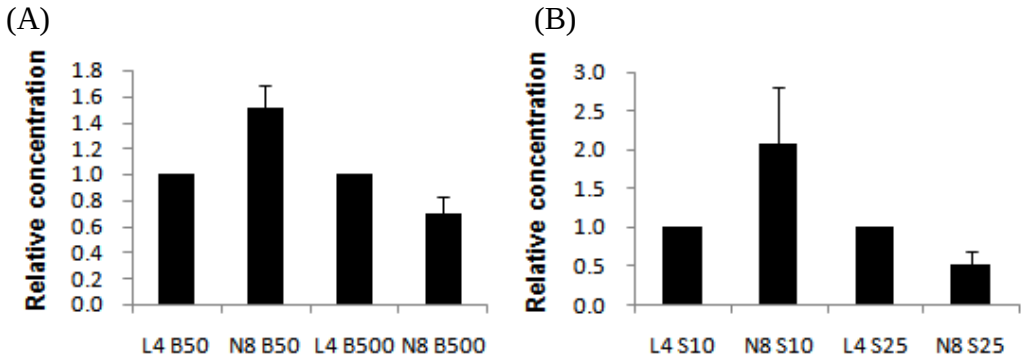

Fig. 3-4 Relative concentration of the accumulation of (A) berberine, (B) sanguinarine and their metabolites (m/z 334, 336) in RNAi control and *nhr-8* RNAi worms. Values are presented relative to the RNAi vector control which was set at 1 for each concentration tested. These values represent the average of three independent experiments. Error bars = SD. L4– RNAi vector control, N8 – *nhr-8* RNAi worm, B – berberine, S – sanguinarine.

The major detoxification response genes induced by berberine and sanguinarine are mostly* NHR-8 *independent

Enhanced sensitivity of the *nhr-8* RNAi worms to the lipid reduction effects of berberine and sanguinarine and higher accumulation of alkaloids in the worms suggest that the xenobiotic response in NHR-8 knockdown worms may have been impeded. To clarify the actual target of NHR-8, the expressions of the major detoxification genes which were up-regulated after berberine and sanguinarine treatment in wild-type worms, were analyzed in *nhr-8* RNAi worms. However, qRT- PCR analysis showed similar increase of gene expression in *nhr-8* RNAi worms after berberine and sanguinarine treatment as in wild-type worms (Fig. 3-5).

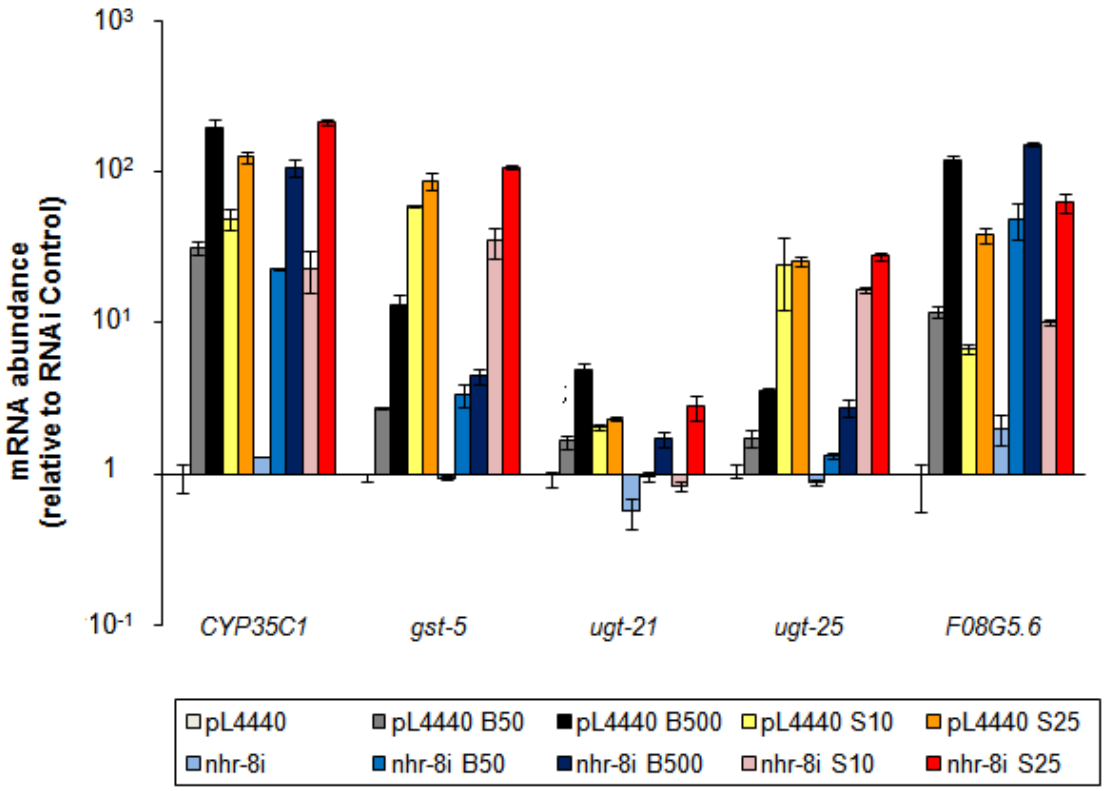

Fig. 3-5 Expression of detoxification genes in *nhr-8* RNAi worms after alkaloid treatment. qRT-PCR showed the expression of. The mRNA abundance value represents the average of three independent experiments (each experiment with triplicate cDNA template applied to run qRT-PCR and the results were normalized against *cdc-42*). Values are presented relative to the RNAi vector control which was set at 1. *pL4440* – RNAi vector control, *nhr-8i* – *nhr-8* RNAi worm, B – berberine, S – sanguinarine.

Differences in induction of xenobiotics response by a wide range of chemicals have been found in *C. elegans* indicating its complexity as well as specificity. Such complex response is likely to be regulated by more than a single PXR/CAR-like regulator (NHR-8). The mediator subunit MDT-15, a transcriptional coregulator in RNA polymerase II dependent transcription, appears to regulate a number of *nhr-8* independent genes encoding cellular metabolic enzymes including CYPs, UGTs and GSTs in response to fluoranthene but not β-naphthoflavone (Kimura et al, 1997). Taubert et al. (2008) 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 MDT-15

dependent expression of those genes. Other transcription factors may also regulate some xenobiotic responses such as *skn-1,* the *C. elegans* homologue of Cap 'n' Collar transcriptional regulator of phase II detoxification and the oxidative stress response (Shore and Ruvkun, 2013).

The qRT-PCR results (Fig. 3-5) also confirmed that the major detoxification genes up-regulated after alkaloid treatment were NHR-8-independent. Interestingly, the expression level of *ugt-21* was reduced in *nhr-8* RNAi control, berberine- and sanguinarine-treated worms. More detailed studies are needed to determine the target genes of NHR-8 although *ugt-21* could be one of them. Different sensitivity to berberine and sanguinarine treatment in *nhr-8* RNAi worms would be useful to elucidate the target genes of NHR-8.

Although the effect of berberine was enhanced in *nhr-8* RNAi worms, its effect on their mobility and mortality was indistinguishable within 24 hours treatment. Since *C. elegans* has extensive and large number of detoxification genes as shown in Fig. 3-6, the effect of NHR-8 knockdown would be marginal on the total detoxification processes.

Berberine is reported to be substrate of the multidrug membrane transporter, P-glycoprotein (P-gp) and are readily effluxed, thus resulting in low bioavailability (Wang et al., 2009; Maeng et al., 2002). Increase in berberine accumulation (at 50 μM) in *nhr-8* RNAi worms suggests that P-gp function may be impeded. On the other hand, sanguinarine acts as a P-gp mediated multidrug resistance reversal agent and it inhibits ATP-binding cassette (ABC) transporter activity (Eid et al., 2012b), suggesting the higher bioavailability and effectiveness of sanguinarine at lower concentration (25 μM) in comparison with berberine (500 μM). More detailed studies of the ABC transporters in *C. elegans* would be useful to elucidate the NHR-8-dependent detoxification process and to develop more efficient bioassay systems.

Overall Summary

One of the advantages in using *C. elegans* is that about 40% of its genes associated with human therapeutical targets have homologs in the worm, making it a good model to study drug-target interaction and target validation (Culetto and Sattelle, 2000). Once an activity is found, forward and/or reverse genetics can be applied to elucidate the possible cellular targets or mechanism of activation.

In Chapter I, preliminary screening on lipid reduction activity of chemicals in *C. elegans* was carried out by staining method to observe the effects through its transparent body wall. The positive results of lipid reduction activities was extrapolated to find out if the effect is linked to AMPK signaling pathway as was reported for berberine through immunoblotting and gene knockdown experiments. Most of the reported studies on AMPK activation validation utilized liver cells of rodents or other mammalian model and this study showed that *C. elegans* can also be applied for such studies. These are advantageous factors for bioactivity screening endeavor as it expands the scope of worm application in multiple assays beyond preliminary screen. Promising candidate compounds can be further validated in mammalian models at a later stage and could thereby bring down the cost of drug development due to the economical benefits in using a worm model. In this study, berberine and sanguinarine were found to have lipid-reducing activity which is linked to the AMPK activation. This activity was dependent on AAK-2, the *C. elegans* homologs of the catalytic α-subunits of AMPK. On the other hand, experiment using *nhr-49* RNAi worms showed that the lipid reduction activity of both alkaloids act independently from *nhr-49* regulated fatty acid β-oxidation process. Furthermore, RNAi control worms treated with berberine and sanguinarine showed reduced *nhr-49* expression which suggests that they have antagonistic effect on lipid synthesis and degradation processes. However, these alkaloids predominantly affect lipid synthesis instead of fatty acid β–oxidation process leading to the overall lipid reduction observed in the worms.

Although both berberine and sanguinarine reduced lipid accumulation in *C. elegans*, several different characteristics of the two alkaloids were noted. Berberine, a protopine-type of alkaloid, required a relatively high effective dosage (500 μM) for its lipid-

reducing activity. The phosphorylation level of the worm's AMPK α-subunit was weaker with berberine compared to sanguinarine after 24 h treatment. On the other hand, sanguinarine, a benzophenanthridine-type of alkaloid, showed more potent lipid-reducing activity with an effective dosage at 25 μM. Stronger AMPK α-subunit phosphorylation activity was observed with sanguinarine-treated worm after 24 h.

In Chapter II, the established assay was extended to evaluate the effects of mixed secondary metabolites in plant cell cultures on lipid reduction. The results showed that *C. japonica* cell culture extract which contains berberine as major component had stronger lipid reducing activity than berberine at similar concentration. On the other hand, *E. californica* cell culture extract with its major component sanguinarine showed weaker activity than in single compound treatment. This shows that cell extracts contain both synergistic and antagonistic components that may lead to differences in the bioactivity strength compared to single alkaloids. In addition to single compound, mixtures also possess bioactivities that are pertinent to new drug development. This simple comparison of pure compounds and crude cell extracts would be useful to characterize the bioactive composition in natural medicine. The rapid advancement in the fields of plant metabolic engineering and synthetic biology would supply more potent materials for the development of new medicine (Sato et al., 2001; Minami et al, 2008).

In Chapter III, I developed a more sensitive *C. elegans* bioassay system since this worm has extensive physical, such as its thick cuticle, and molecular (detoxification enzymes) defense mechanisms against xenobiotics. Although the effective dose of test compounds in worm was at higher orders of magnitude than that in mammalian cell culture (Kwok et al., 2006; Broeks et al., 1995), *nhr-8* RNAi worms were found to be more susceptible to the lipid reduction effects of some isoquinoline alkaloids, especially berberine which showed increased sensitivity by 8-fold. Increased sensitivity to alkaloid treatment in *nhr-8* RNAi worms suggests that such detoxification mechanism may be impeded. However, the major upregulated detoxification genes were found not impeded, except for *ugt-21* which could be a target of NHR-8. This *nhr-8* RNAi worms would be especially useful for screening of complex plant alkaloids whereby the amount isolated for testing are scarce.

This collective studies show that *C. elegans* is a versatile, whole organism probe for isoquinoline alkaloids bioactivities screening. Using this worm bioassay system, the potent lipid modulation activity in certain classes of alkaloids and the probable action mechanisms were revealed. The established assays could be extended to screen bioactivities of more structurally diverse alkaloids as well as mixture of plant metabolites in cell cultures in the future. This live worm model also enables concurrent monitoring of the alkaloids' toxic effect. Such bioassay system is useful for new drug leads and drug-target investigations.

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Chow YL, Sato F. (2013) Screening of Isoquinoline Alkaloids for Potent Lipid Metabolism Modulation with *Caenorhabditis elegans*. *Biosci Biotechnol Biochem.* 77(12): 2405-2412.

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