Running title: IQA Screen for Lipid Metabolism Modulation with *C. elegans*

Screening of Isoquinoline Alkaloids for Potent Lipid Metabolism Modulation with *Caenorhabditis elegans*

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Abbreviations: ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; IQA, isoquinoline alkaloid; RNAi, RNA interference; NHR, nuclear hormone receptor

Metabolic syndrome and related disorders are increasingly prevalent in contemporary society, and thus pose the need for potent agents to control lipid accumulation in the body. This study indicates that *Caenorhabditis elegans* was effective in screening for potent lipid metabolism modulators with berberine as a model compound. Among the various isoquinoline alkaloids tested, sanguinarine, a benzophenanthridine alkaloid, was found to be the most potent. Sanguinarine, like berberine, reduced lipid accumulation through AMP-activated protein kinase activation. Analysis of AMPK (*aak-1* and *aak-2*) RNAi worms revealed that effects were *aak-2*-dependent. Characterization of worms with knockdown *nhr-49*, a hormone nuclear receptor gene that functions as a key regulator of fat consumption, showed that both alkaloids were effective even in these markedly lipid-accumulating *nhr-49* RNAi worms, suggesting that they predominantly affect lipid synthesis, rather than fatty acid β-oxidation. The versatility of *C. elegans* for the purpose of lipid-modulating chemical screening and characterization of the underlying mechanisms is discussed.

Key words: berberine; sanguinarine; lipid metabolism; AMP-activated protein kinase; *Caenorhabditis elegans*

Risk factors for metabolic syndrome, such as obesity, diabetes, and high blood pressure, are major concerns in modern society. To help reduce these health risks, many studies have been done to identify agents that can potently control the accumulation of lipids, which mediate metabolic syndrome. Several natural compounds, including catechin in green tea and capsaicin in chili pepper, have been reported to be good candidates.[1, 2)]

In this study, we screened for more potent candidates among alkaloids, which exhibit strong pharmacological activities, and particularly isoquinoline alkaloids. Berberine, a benzylisoquinoline alkaloid that can be obtained from *Berberis* (Berberidaccae) and *Coptis* rhizomes, is used as an antibacterial agent in the treatment of gut infections and diarrhea. Recent reports on decreased plasma cholesterol and triglyceride levels in hypercholesterolemic patients, and reduced body weight and plasma triglyceride levels and improved insulin action in rats fed high-fat diets under treatment with berberine, suggest that it can be useful as a cholesterol-lowering drug.[3)] Furthermore, recent advances in the metabolic engineering of isoquinoline alkaloid biosynthesis, including both synthetic biology and chemical synthesis, have indicated that it might be possible to produce a wide array of alkaloids of this type.[4, 5)]

In this study, we used *Caenorhabditis elegans* as a live model to detect the biological activities of isoquinoline alkaloids, since this worm offers several distinct advantages in preliminary screening, in terms of cost, time, and ease of manipulation, as well as effectiveness in gene-to-drug screening.[6, 7)] Furthermore, an intact animal offers advantages over *in vitro* or cell-based assays for subsequent application to the whole animal.[8)] In addition, *C. elegans* largely shares the core mechanisms of the regulatory pathways of mammalian energy homeostasis. The usefulness of *C. elegans* in screening small molecules to identify new regulators of fat storage has been demonstrated by Lemieux *et al*.[9)]

Initially, we validated this worm bioassay system with berberine as a model

modulator of lipid metabolism, and further screened several isoquinoline alkaloids for lipid metabolism-modulation activity. Among the isoquinoline alkaloids examined, sanguinarine had a greater effect on lipid accumulation than berberine. Since there are no previous reports to the effect that sanguinarine affects lipid metabolism or the insulin signaling pathway, except for a recent report that it directly activated AMP-activated protein kinase (AMPK),[10] we examined the mechanism by which sanguinarine reduces lipid levels through the AMPK pathway, since there are various AMPK activators and AMPK activation mechanisms.

AMPK functions as a fuel gauge, as it were, in biological systems, and is a prime target for new therapies for metabolic disorders, such as insulin resistance and type 2 diabetes.[11] It is activated under conditions that deplete cellular ATP and elevate AMP levels. Recent studies have found that AMPK can also be activated by other stimuli that do not cause any detectable change in the AMP/ATP ratio, such as hyperosmotic stress and pharmacological agents like thiazolidinediones (TZD), metformin, and 5-aminoimidazole-4-carboxamide ribotide (AICAR). Upon activation, AMPK phosphorylates and inactivates several metabolic enzymes, that are involved in ATP-consuming pathways, including acetyl CoA carboxylase (ACC), fatty acid synthase, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, and the mammalian target of rapamycin (mTOR), and activates ATP-generating processes such as fatty acid oxidation and glucose uptake (Supplemental Fig. 1; see *Biosci. Biotechnol. Biochem.* Web site).

Since decreased lipid synthesis and increased lipid consumption in vivo should reduce lipid accumulation, we further investigated the involvement of fatty acid oxidation in the lipid-reducing effects of berberine and sanguinarine. The gene for a key regulator of fat consumption in *C. elegans*, *nhr-49*,[12] was knocked down, and the effects of berberine and sanguinarine were examined.

Materials and Methods

Chemicals and reagents. Berberine sulfate was purchased from Tokyo Chemical Industry (Tokyo, Japan); AICAR from Wako Pure Chemicals (Osaka, Japan); sanguinarine chloride, alpha lipoic acid, and cholesterol from Sigma-Aldrich Japan (Tokyo, Japan); and magnoflorine, aromoline, tetrandrine, and isotetrandrine were the kind gift of Dr. K. Iwasa (Kobe Pharmaceutical University). Phospho-Thr172-AMPK and AMPK antibodies were from Cell Signaling Technology (Danvers, MA), and horseradish peroxide-conjugated donkey anti-rabbit IgG, from GE Healthcare Japan (Hino, Japan). 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 by standard culture methods.[13)]

Nematode treatment. In preliminary screen by Nile Red staining, 3-d-old worms were used. When larger amounts of worms were required for Oil Red staining, and protein and RNA extraction, 2-d-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 24 h of treatment.

Nile Red staining. Synchronized 3-d-old *C. elegans* were treated with various chemicals for 24 h on NGM medium plated with *E. coli* OP50 as food source and 50 ng mL^{-1} of Nile Red (Sigma) 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 h of culture at 20°C, 10 to 12 worms were randomly selected for observation against a bright field, and red fluorescence images were obtained by the Keyence BIOREVO BZ-9000 Imaging System (Keyence, Osaka, Japan). Hazy images were omitted, and the rest were used to quantify the fluorescence intensity of lipid droplets with 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 of one experiment.

Oil Red O staining. Two-d-old worms were treated with and without alkaloids (as control) for 24 h as in the Nile Red staining experiments. About 200-300 worms were collected and washed 3 times with 1X PBS pH 7.4 buffer. Oil Red O staining was done as reported by O' Rourke *et al*.[14] After staining, 10 to 12 worms were selected randomly for observation under a bright field, and images were captured with a Keyence BIOREVO BZ-9000 Imaging System (Keyence). Hazy images were omitted, and the remaining ones were used to quantify the intensity of Oil Red O staining with ImageJ software. Each color image was separated into its RGB channel components, and the green channel was used for further analysis following the method of Yen *et al*.[15] The results were verified by reproducibility in at least two of three independent experiments. The figures represent the results of one experiment.

RNA Interference. An RNAi feeding method[16] was used. Some 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'; and *nhr-49* reverse, 5'-AGAGGATGAATTGCCAATGGAG-3'.

The primers were fused with restriction enzyme sites. Xba1 and Xho1 for *aak-1* and *aak-2,* and Xba1 and Xma1 for *nhr-49* and were used to amplify the genes of interest. Each cDNA segment was cloned into feeding vector pL4440 (kind gift of Dr.

A. Fire, Stanford University) with the various restriction sites and transformed into HT115 bacterial cells. Colonies were screened, and positive transformants were confirmed by PCR with the original primer pairs. RNAi control worms were fed bacteria carrying an empty pL4440 vector. The details of the procedures for RNAi treatment are described in Supplemental Information.

Immunoblot analysis. Two-d-old *C. elegans* were treated with 1 mM of various chemicals for 24 h on NGM medium plated with *E. coli* OP50 as food source. Procedures for immunoblot analysis are described in Supplemental Information.

Quantitative RT–PCR. Total RNA was extracted with Sepasol-RNA I Super G (Nacalai Tesque, Kyoto, Japan), purified with an RNeasy Mini Kit (Qiagen Japan, Tokyo, Japan), and reverse-transcribed into cDNA with SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) with oligo(dT) primer. cDNA (final concentration, 500 pg μL^{-1}) was subjected to qRT-PCR analysis by the CFX96 Real-Time PCR System (Bio-Rad, Hercules, CA) with IQ SYBR Green Super Mix (Bio-Rad). The conditions for the PCR reactions were 95°C for 15 min, followed by 40 cycles of 95 °C for 10 s, 60°C for 20 s, and 72°C for 20 s. Melting curve analysis was done after each run at 72°C to 95°C to check the specificity of amplification. Data were analyzed with 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 amplification of *cdc-42* as internal control.

Sequences of forward and reverse primers used in quantitative RT-PCR analysis were as follows:
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'; and *cdc-42* reverse, 5'-CTCGAGCATTCCTGGATCAT-3'.

Statistical analysis. Statistical comparisons between groups were evaluated by ANOVA, followed by Dunnett`s multiple comparisons test for measurements of Nile Red fluorescence and Oil Red O staining intensity, and for qRT-PCR analysis. Differences were considered to be significant at $p < 0.05$. Error bars represent standard deviation unless otherwise stated.

Results and Discussion

Nile Red and Oil Red O staining revealed the effects of isoquinoline alkaloids on lipid accumulation in the treated worms

Fig. 1 and 2

In preliminary experiments, we examined the effect of berberine, a known lipid modulator, by Nile Red staining[9,17] and confirmed that it has a lipid-reducing effect. We further validated the effect of berberine by Oil Red O stain, since it is a more accurate proxy for major fat stores in the subcellular compartments of the worm.[14]

Next we examined the effects of several other isoquinoline alkaloids (sanguinarine, magnoflorine, isotetrandrine, tetrandrine, and aromoline), by Nile Red stain. AICAR, an AMP mimetic nucleoside commonly used to activate AMPK, and alpha lipoic acid, which improves hypertriglyceridemia by stimulating triacylglycerol clearance and downregulating liver triacylglycerol secretion, were selected as reference lipid-reducing agents.[18,19]

Treatment of worms with berberine, sanguinarine, and AICAR at 1 mM

significantly reduced lipid accumulation, while treatment with alpha lipoic acid only slightly reduced lipid accumulation. The other alkaloids produced little or no reduction in fluorescence at concentrations of up to 5 mM (Fig. 2A and B). All the worms showed normal growth and mobility during the 24-h treatment period.

The lipid-reducing effect of sanguinarine was further verified by Oil Red O stain (Fig. 2C and D). Due to the limited availability of some chemicals, we confirmed only that magnoflorine and tetrandrine did not have lipid-reducing effects by Oil Red O stain (Supplemental Fig. 2). Sanguinarine exhibited much stronger lipid-reducing activity than berberine. It significantly reduced lipid accumulation at a concentration (25 μM) almost 20-fold lower than the effective concentration of berberine (Fig. 3 and Supplemental Fig. 3).

Sanguinarine induced phosphorylation of AMPK Fig. 3

The AMPK pathway has been reported to be involved in the regulation of fat metabolism for berberine-induced fat reduction,[18] in which the inhibition of ACC in HepG2 cells resulted in inhibition of cholesterol and triglyceride synthesis.[20] We further characterized the effects of sanguinarine on AMPK and its downstream molecules in comparison with the effects of berberine.

To determine the conditions for activation of AMPK, we checked its phosphorylation conditions with anti-phospho AMPK (pAMPK)-specific antibody. Treatment with sanguinarine and AICAR induced the phosphorylation of AMPK (Fig. 4), whereas berberine had only a marginal effect, but phosphorylation of AMPK by treatment with berberine has been reported.[20,21] A possible explanation for the weak phosphorylation detected in our experiments is the lower reaction specificity of the antibody against *C. elegans* proteins, since previously we detected phosphorylation of AMPK induced by berberine in HepG2 cells (data not shown).

Knockdown of the C. elegans *homologs of catalytic α-subunits of AMPK revealed* Fig. 4

that aak-2 *is involved in the mechanisms by which berberine and sanguinarine modulate lipids*

To investigate the mechanisms by which berberine and sanguinarine reduced lipid levels in the treated worms, we knocked down the genes encoding the *C. elegans* homologs of the catalytic α-subunits of AMPK, *aak-1,* and *aak-2*. AAK-1 and AAK-2 are 40% and 52% identical to and share 71% and 80% amino acid identity respectively with the kinase domain of human AMPKα subunits. They also contain a conserved critical threonine residue, the phosphorylation of which is required for AMPK activation.[11] The function of AAK-1 has not been much characterized, except for a study by Lemieux *et al*.[9] in which they identified an undisclosed compound, F17, that activated AMPK signaling in *C. elegans via aak-1*.

When *aak-1* or *aak-2* was knocked down by RNA interference (RNAi) with double-stranded RNA of these genes, lipid levels were reduced by berberine and sanguinarine in the *aak-1* RNAi worms, while there was little response in the *aak-2* RNAi worms (Fig. 5A and B), which suggests that AMPK, and especially *aak-2*, plays an important role in the lipid reduction induced by berberine and sanguinarine. Quantitative real-time PCR (qRT-PCR) analysis of the transcripts of *aak-1* and *aak-2* in the *aak-1* RNAi worms indicated that *aak-2* expression was induced roughly in proportion to reduction in *aak-1* (Supplemental Fig. 4A). On the other hand, the *aak-1* transcript level was unaffected in the *aak-2* RNAi worms (Supplemental Fig. 4B). These results suggest that AMPKα subunit expression in the worms was regulated mainly by *aak-2,* that accounts for most of the basal activity and activation by berberine and sanguinarine. Li *et al*.[22] have reported that the α1 and α2 isoforms of the catalytic AMPK subunits in most tissues of the ischemic rat heart can be differentially activated due to different sensitivities to small changes in the AMP concentration. Our results suggest that in this worm, the *aak-1* isoform of the AMPKα subunit is less responsive to activation by berberine and sanguinarine.

Fig. 5

Sanguinarine was effective at reducing lipid levels in the high-fat worms with a deficient β-oxidation pathway

While nuclear hormone receptor NHR-49 in *C. elegans* is highly homologous to hepatocyte nuclear factor 4 (HNF4) in mammals, it is functionally more similar to mammalian peroxisome proliferator-activated receptors (PPARs). NHR-49 modulates fat-consumption pathways as a key regulator of fat usage, and maintains the fatty acid saturation in balance. It targets multiple enzymes involved in transporting fatty acids across the mitochondrial membrane for β-oxidation. Hence, *nhr-49* knockout exhibited a high-fat phenotype due to deficiencies in two metabolic pathways: fatty acid β-oxidation and fatty acid desaturation.[12)]

We conducted an RNAi experiment on *nhr-49* and investigated the involvement of β-oxidation in lipid-reduction by berberine and sanguinarine. Consistently with the reported high-fat phenotype, the *nhr-49i* worms showed increased fat storage under Oil Red O staining (Fig. 6A and B, Supplemental Fig. 5). They showed a marked increase in body size and significantly lower expression of mitochondrial β-oxidation genes, *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. 6C). NHRs respond to lipophilic molecules, regulating the expression of target genes involved in metabolism, reproduction, and development.[17)] Liang *et al*.[23)] have reported that NHR-64, another *C. elegans* HNF4 nuclear receptor, regulates fat storage, growth, and brood size in these worms.

Despite their high fat accumulation, the *nhr-49* knockdown worms were sensitive to berberine and sanguinarine, suggesting that both alkaloids act independently of *nhr-49* (Fig. 6A). Unexpectedly, an analysis of *nhr-49* transcript levels in RNAi control worms (*pL4440*) treated with berberine and sanguinarine showed reduced *nhr-49* expression (Fig. 6D), albeit to a lesser extent than for *nhr-49* RNAi. While the details of the antagonistic action of berberine and sanguinarine on

Fig. 6

lipid synthesis (*via* AMPK) and degradation (*via* NHR-49) are not clear, inhibition of lipid biosynthesis through AMPK activation had a greater effect on lipid accumulation.

Effects of plant alkaloids on lipid modulation and the mechanisms

In our primary screening of isoquinoline alkaloids, protoberberine (berberine)- and benzophenanthridine (sanguinarine)- type compounds showed lipid-reducing activity, while other types of isoquinoline alkaloids, including aporphine (magnoflorine)- and bisbenzylisoquinoline (isotetrandrine, tetrandrine, aromoline)-ones, did not. Screening of metabolically modified plant cell extracts consisting of protoberberine and benzophenanthridine alkaloids can provide useful information on the most bioactive alkaloids as drug leads. Preliminary screening of plant cell extracts in the ongoing study suggested that a cell extract is more potent than a single compound such as sanguinarine (data not shown). Cell extracts can have multiple cellular targets and effects that are probably due to multiple interactions of the quaternary nitrogen, and polycyclic and planar structures with nucleophilic and anionic moieties of amino acids in biomacromolecules.[24)]

Whereas both berberine and sanguinarine are potent lipid modulators, these two compounds have different chemical structures and points of efficacy. Our biochemical and RNAi experiments indicate that their effects are dependent on AMPK and enhanced AMPK phosphorylation, which control several metabolic processes including lipid metabolism. AMPK is stimulated by AMP through allosteric activation of phosphorylated AMPK and by inhibition of the dephosphorylation of its Thr-172 by protein phosphatases.[25)] These effects are due to the binding of AMP at two exchangeable sites on the γ- subunits, and are opposed by ATP, which binds at the same sites. AMPK activation might also be the result of direct binding to AMPK at sites distinct from that of AMP, as reported for chemicals such as A769662 and salicylic acid.[26)]

Plant alkaloids perhaps defend against herbivores and pathogens. Many alkaloids have been reported to be mitochondrial inhibitors and might also be potent AMPK activators.[27] Activation of AMPK by berberine and sanguinarine might be the result of inhibition of ATP synthesis and an increase in the AMP level based on the finding that these alkaloids inhibited mitochondrial respiration.[21] Studies of cell lines that express AMPK complexes bearing an R531G mutation, which renders γ2 complexes insensitive to increases in ADP and AMP, have indicated that berberine works by increasing cellular AMP or ADP. On the other hand, Choi *et al*.[10] reported that sanguinarine directly interacted with AMPK and enhanced its activity to a level above that mediated by AMP.

It would be interesting to investigate the difference between indirect and direct AMPK activation and its implications for the potency of alkaloid bioactivity. Although both berberine and sanguinarine activate AMPK, the difference in the uptake efficiency of these chemicals should also be considered, since Turner *et al*.[21] have reported greater absorption of dihydroberberine as compared to berberine due to the structural modification.

Besides reductions in lipid synthesis, increments in lipid consumption might also decrease lipid accumulation in an organism. We investigated this with *nhr-49* knockdown worms. Our *nhr-49* RNAi experiment showed reduced lipid accumulation under berberine and sanguinarine treatment (Fig. 6A), while *nhr-49* expression was also reduced in the control worms treated with both alkaloids (Fig. 6D). NHR-49 acts as a positive regulator of *acs-2* and *ech-1* promoting fatty acid oxidation by mitochondria. Pathare *et al*. [28] have reported that NHR-49 activated and repressed genes in multiple lipid metabolism pathways. Thus knockdown of *nhr-49* can lead to high lipid accumulation (Fig. 6A and B). Our results suggest that berberine and sanguinarine have antagonistic actions on and lipid synthesis (*via* AMPK activation) and oxidation (*via* NHR-49 downregulation) processes. The stronger effects of sanguinarine and berberine treatment in lipid reduction suggest

that lipid biosynthesis regulated by AMPK activation predominates over the downregulated lipid degradation process due to *nhr-49* (Supplemental Fig. 1).

Versatility of the worm

The results of this study confirm the feasibility of using *C. elegans* for bioactivity screening of plant isoquinoline alkaloids. One of the advantages of *C. elegans* is that about 40% of the genes associated with human therapeutical targets have homologs in the worm, which makes it a good model for studying drug-target interaction and target validation. This worm has been instrumental in elucidating important aspects of several important receptor-based pathways that regulate energy storage and utilization, including insulin, serotonin, TGF-β, and adiponectin. [29)]

We have used a combination of genetic and biochemical approaches to characterize the lipid metabolism modulation effects of plant alkaloids. In this study, we confirmed that the lipid-reducing activity of sanguinarine is linked to the AMPK signaling pathway and not dependent on the NHR-49 pathway, similarly to berberine (Figs. 4-6). Whereas most previous studies of AMPK activation used liver cells of rodents or another mammalian model, our study indicates that *C. elegans* can also be used. These findings are advantageous for bioactivity screening, since they expand the scope of the worm in multiple assays beyond preliminary screening. Promising candidate compounds can be later validated in mammalian models, and this might ultimately reduce the overall cost of drug development.

Although *C. elegans* can serve well as a screening model, the effective concentration of berberine (400 μM) in this worm to reduce lipid accumulation was much higher than the effective concentration (7 μM) reported for a human hepatoma-derived cell, the HepG2 cell.[3)] This difference might be due to the potential difference in the target sites, and also to different absorption and/or degradation ability of those cells for berberine. Sanguinarine showed higher lipid reducing activity than berberine at low concentrations such as 25 μM (Fig. 3). This

concentration is very close to the EC_{50} value (26 to 28 μM) that activated human recombinant AMPK in an *in vitro* assay.[10] Our preliminary experiments with the *nhr-8* RNAi worm with a knocked-down xenobiotic response, which showed enhanced sensitivity to berberine, may provide clues to understanding the differences between the uptake and metabolism of berberine and sanguinarine *in vivo* (Chow and Sato, in preparation).

Moreover, screening in an intact worm makes it possible simultaneously to monitor toxicity, which is not possible in an *in vitro* assay. For instance, besides lipid-reducing effects, berberine and sanguinarine also have cytotoxic activity, similarly to many plant alkaloids. These chemicals were known to form complexes with DNA and RNA, and to inhibit telomerase and topoisomerase.[30] Sanguinarine exhibited both stronger lipid-reducing activity and stronger cytotoxicity (Supplemental Fig. 6). Separation of the cytotoxic effects from the desired lipid-reducing activity for sanguinarine and related alkaloids is crucial for therapeutical applications.

In this study, we screened for bioactivities in pure, isolated compounds. As has been demonstrated in most ethnobotanical or traditional medicine applications, therapeutic effects are often produced by a concoction of plant extracts. Our future studies will incorporate extracts from medicinal plant cells, including metabolically engineered plant cell cultures, to screen for synergistic bioactivities among plant alkaloids.

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Figure legends

Fig. 1. Structures of Test Compounds.

Fig. 2. Effects of Isoquinoline Alkaloids on Lipid Accumulation.

(A) Nile Red staining in treated worms. Berberine, sanguinarine, AICAR, and alpha lipoic acid were administered at 1 mM. Magnoflorine, isotetrandrine, tetrandrine, and aromoline were administered at concentration of 5 mM. Images show typical results in worms treated with the test compounds for 24 h. Magnification is 40× for the top row and 20× for the bottom row (Scale bar, 100 μm); exposure time, 1/25 s; light intensity, 40%. n, 10 each for three independent experiments. (B) Quantification of Nile Red fluorescence intensity in treated worms with ImageJ software; average values are shown for the compounds tested (see "Materials and Methods") and normalized to the control sample. (C) Oil Red O staining in treated worms. Images show typical results for worms treated with berberine or sanguinarine for 24 h. Magnification at 10× (Scale bar, 100 μm). (D) Quantification of Oil Red O staining intensity in treated worms by ImageJ. Average values are shown for the compounds tested and these are normalized to the control sample. $*p < 0.05$ (ANOVA followed by Dunnett`s test) for the (B) and (D). C, control in (B) and (D).

Fig. 3. Effects of Berberine (A) and Sanguinarine (B) on Lipid Accumulation in Worms.

Quantification of Oil Red O staining intensity using ImageJ. Values are averages of 10-12 images for the compounds tested and these are normalized to the control sample; $*p < 0.05$ (ANOVA followed by Dunnett`s test). C, control in (A) and (B).

Fig. 4. Immunoblot Showing the Phosphorylation of AMPK in the Chemically Treated Worms.

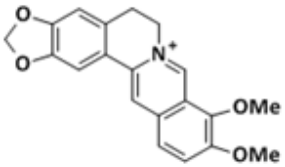
They were treated with berberine, sanguinarine, or AICAR at 1 mM for 24 h. (A) Representative immunoblots of worm protein extracts for anti-phospho AMPKα and anti-AMPKα are shown. (B) Average phosphorylation levels of AMPK for three independent experiments were quantified by ImageJ based on band intensities, and normalized to total AMPK levels in the control worms. *$p < 0.05$ (ANOVA followed by Dunnett`s test). C, control.

Fig. 5. Knockdown of the *C. elegans* Homologs of Catalytic α-Subunits of AMPK.

Relative intensity of Oil Red O-stained lipid droplets quantified by ImageJ in (A) *aak-1* and (B) *aak-2* RNAi worms treated for 24 h with berberine, B at 200 μM or 400 μM and sanguinarine, S at 25 μM. n, 10-12 for the compounds tested; *$p < 0.05$ (ANOVA followed by Dunnett`s test). *pL4440*, RNAi vector control. *aak-1i, aak-1 RNAi worms*. *aak-2i*, *aak-2* RNAi worms.

Fig. 6. Effects of Alkaloid Treatment on *nhr-49* RNAi Worms.

(A) Relative intensity of Oil Red O-stained lipid droplets quantified by ImageJ in *nhr-49* RNAi worms treated with berberine, B (400 μM) or sanguinarine, S (25 μM) for 24 h. C is non-treated control; n, 10-12 for the compounds tested; *$p < 0.05$ (ANOVA followed by Dunnett`s test). (B) Oil Red O staining of RNAi control worms and *nhr-49* RNAi worms. Images show typical results for worms at a magnification of 20× (scale bar, 100 μm). qRT-PCR showed the expression of fatty-acid oxidation genes (C), and *nhr-49* gene (D). The mRNA abundance value represents the average of triplicate cDNA templates applied to qRT-PCR, and the results were normalized to *cdc-42*. $p < 0.05$ (ANOVA followed by Dunnett`s test). *pL4440*, RNAi vector control. *nhr-49i*, *nhr-49* RNAi worms. Results were verified by reproducibility in at least two of three independent experiments.



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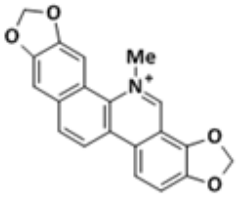

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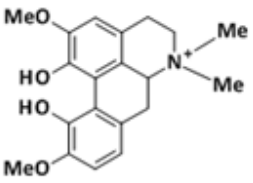

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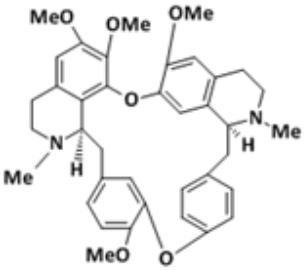

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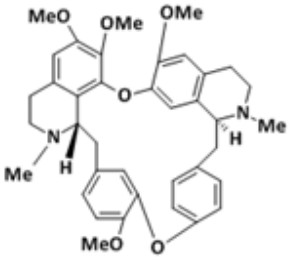

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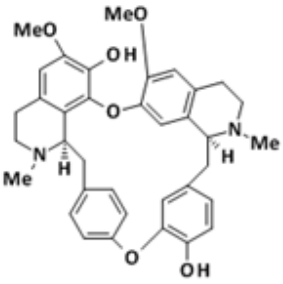

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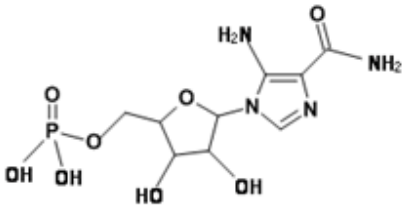

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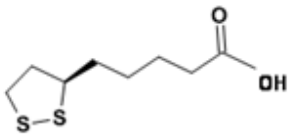
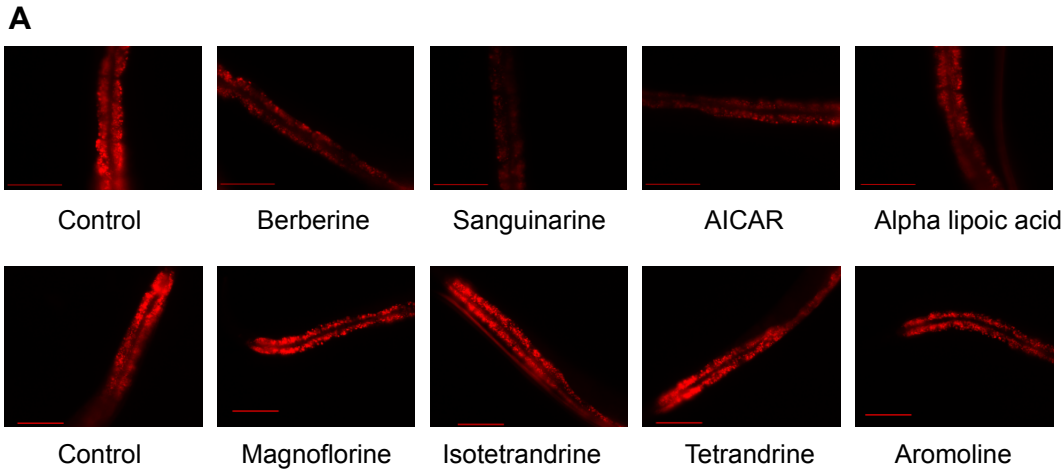
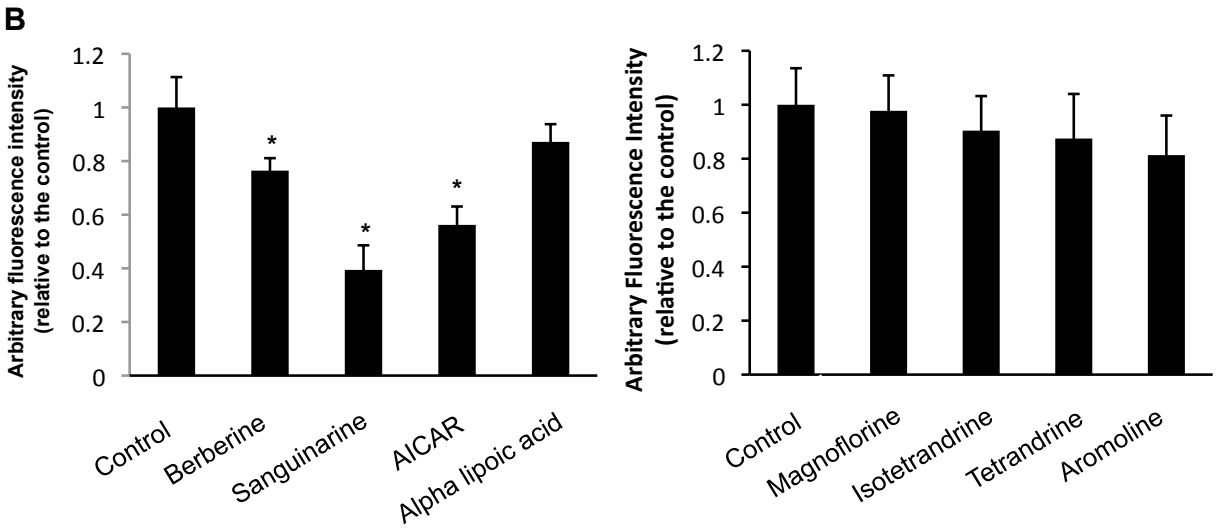
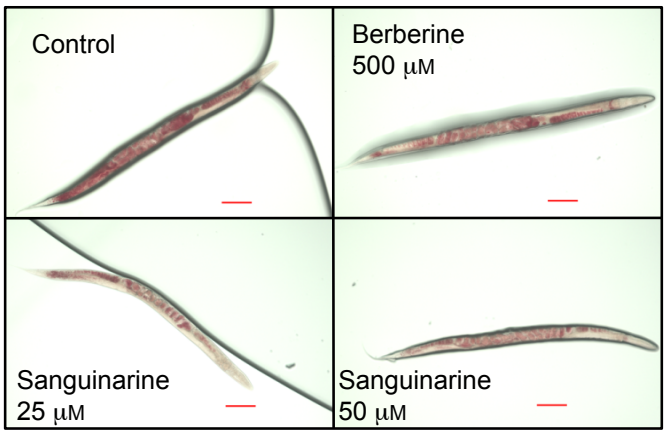


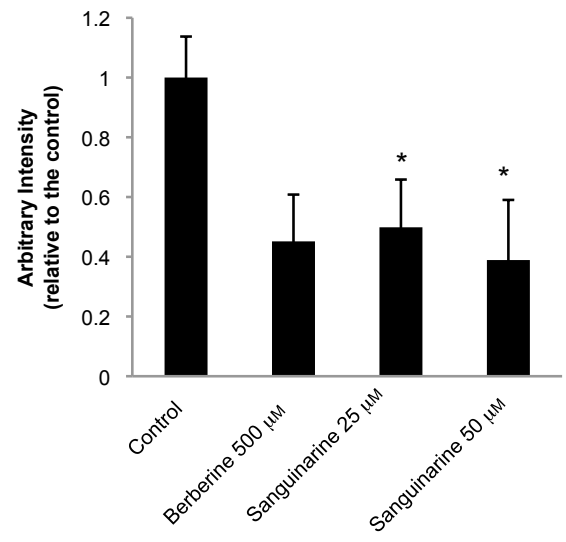
alpha lipoic acid

Figure 1 Chow & Sato









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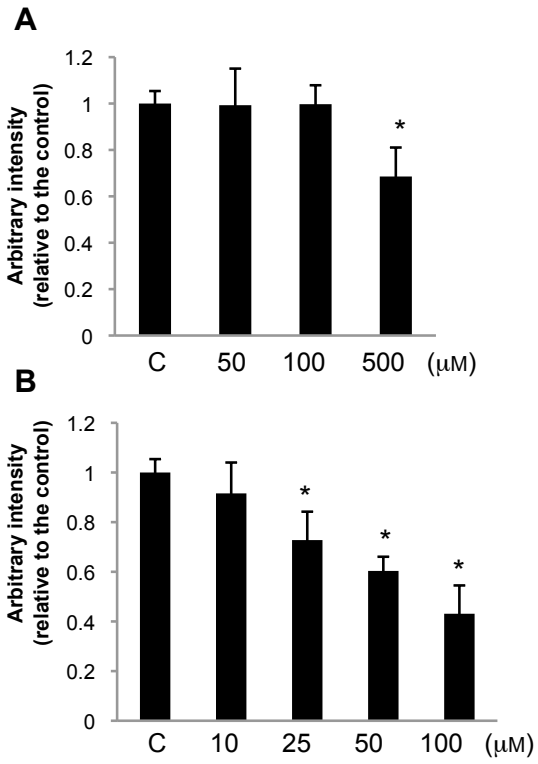

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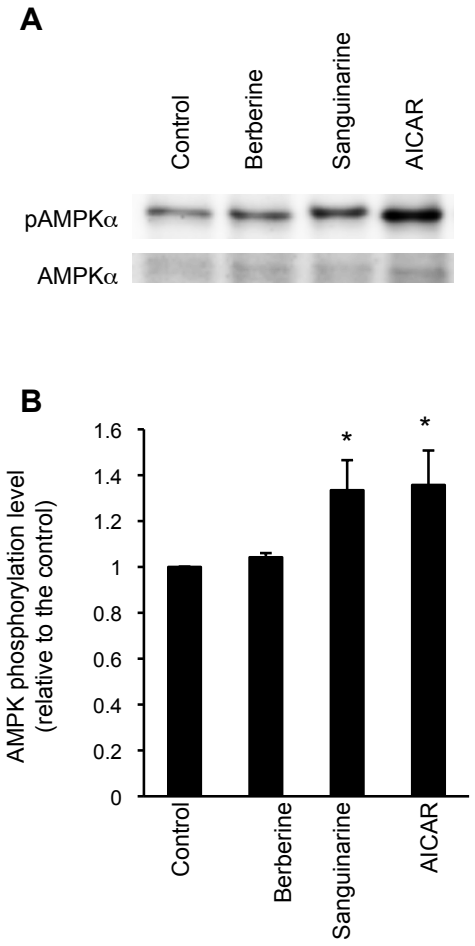

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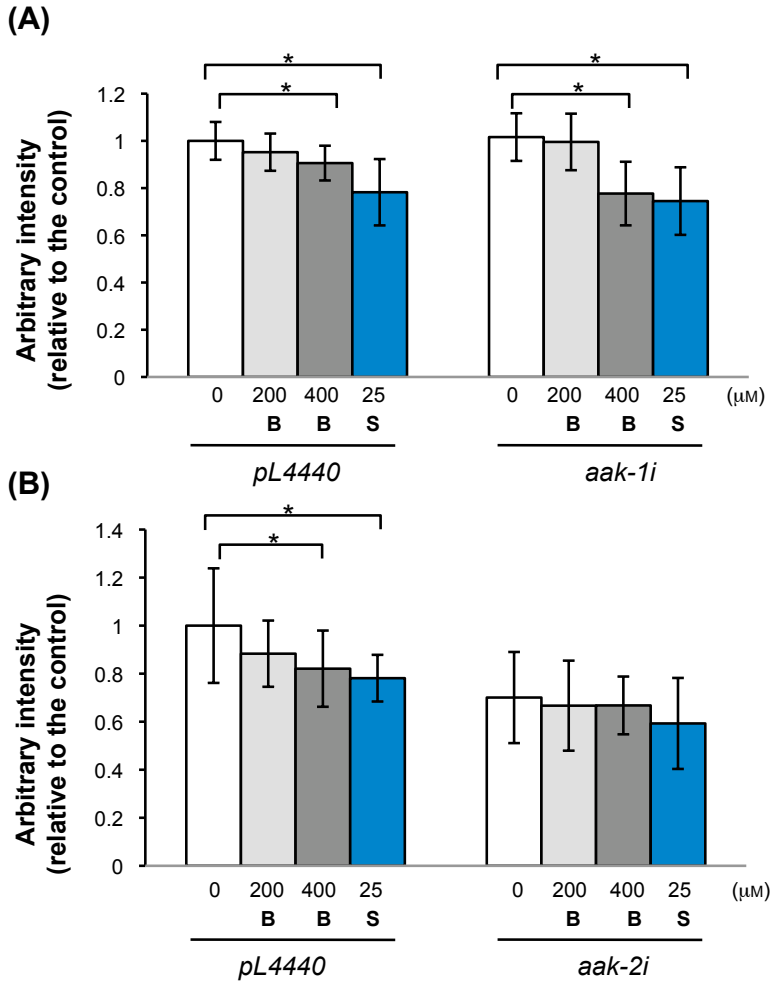

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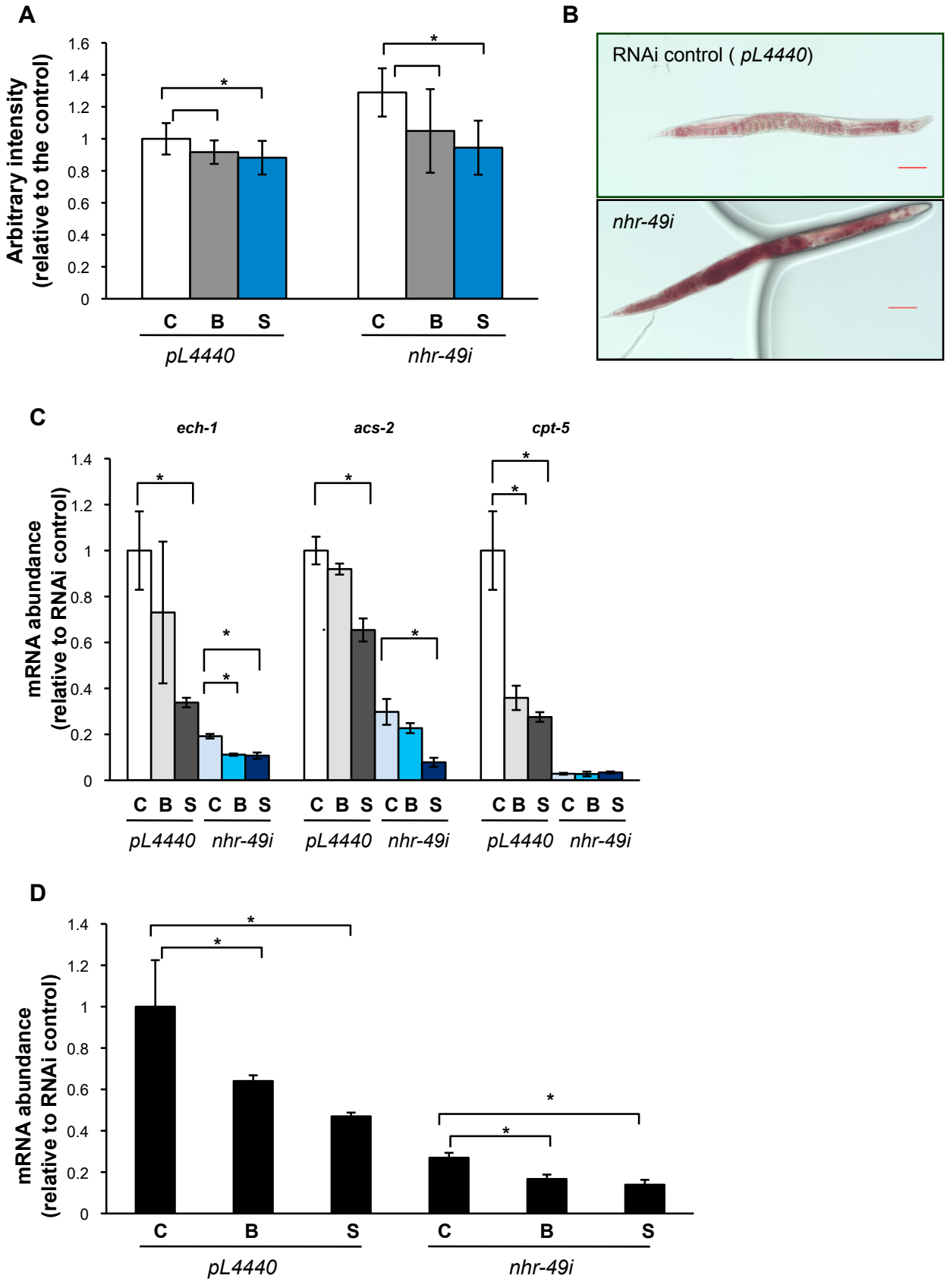

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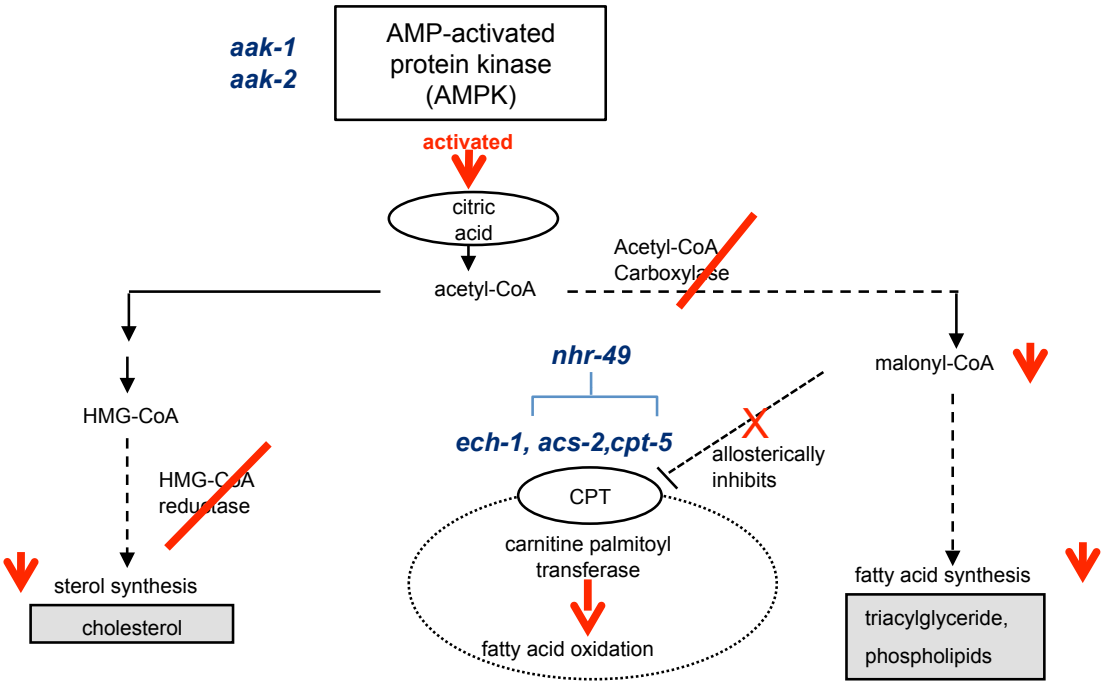
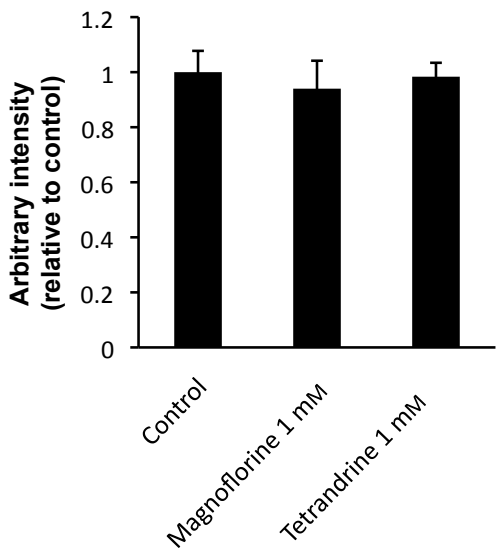


Supplemental Figure 1
Scheme showing the effects of AMPK activation on the lipid metabolism pathway. When AMPK is activated, it phosphorylates and inactivates several metabolic enzymes involved in ATP-consuming pathways such as acetyl-CoA carboxylase (ACC), fatty acid synthase, and 3-hydroxy-3-methylglutaryl-CoA (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 beta 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*.

A

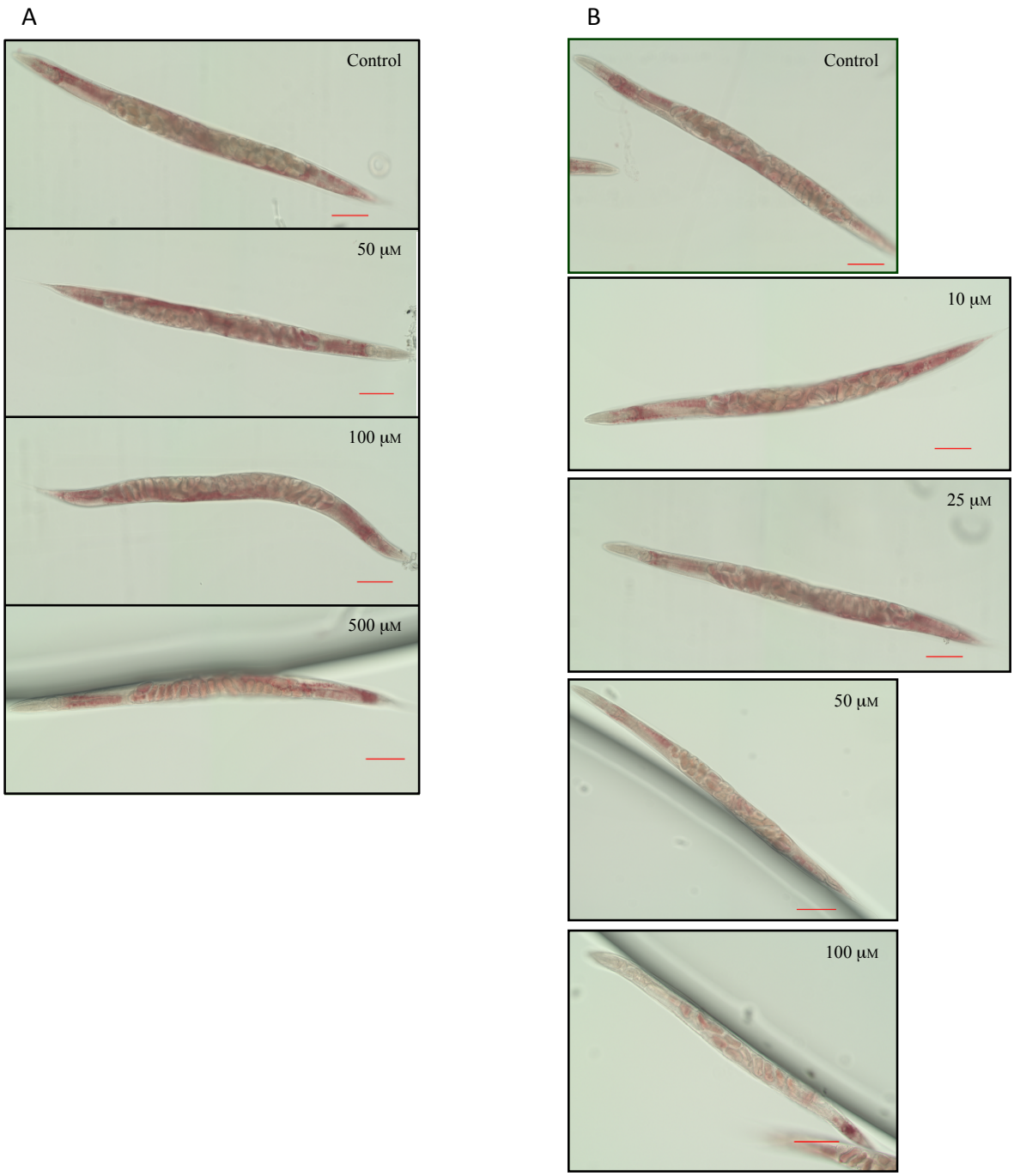
B



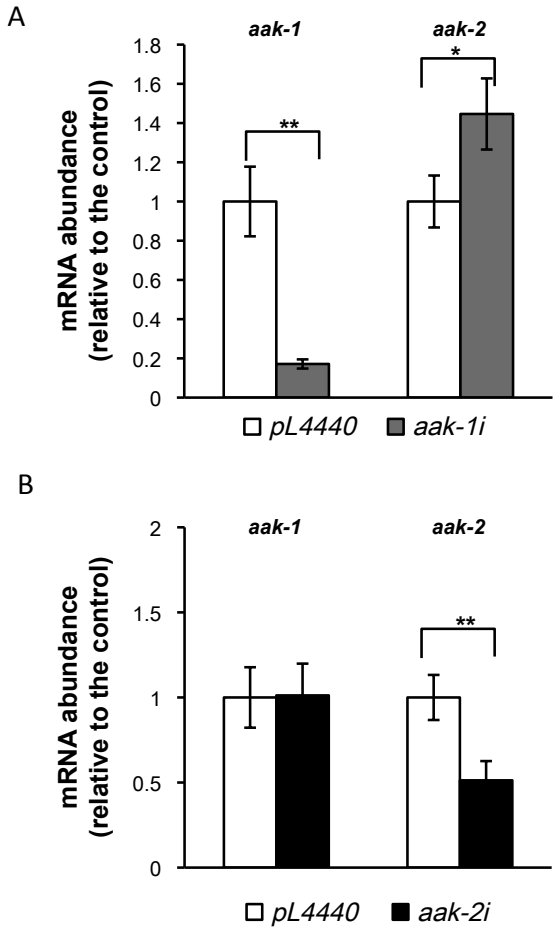
Supplemental Figure 2

(A) Oil Red O staining in treated worms. Images show representative results in worms after treatment with magnoflorine or tetrandrine for 24 h. Magnification at 20x (scale bar, 100 μm). n, 10-12 each from 3 independent experiments.

(B) Quantification of the intensity of Oil Red O staining in treated worms using ImageJ software (http://rsbweb.nih.gov/ij/). Values are the average of 10-12 images for each compound tested and are normalized to the control sample.

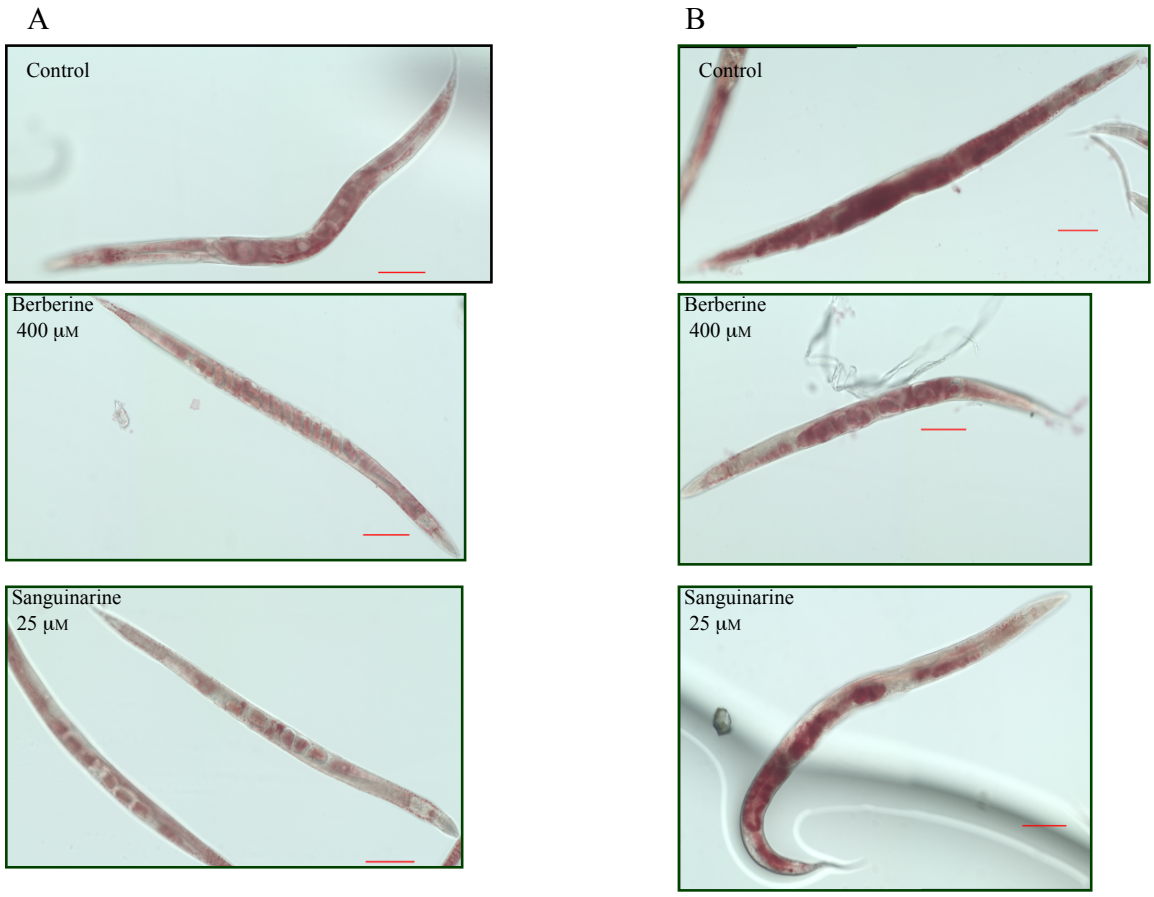


Supplemental Figure 3
Oil Red O staining in worms treated with (A) berberine and (B) sanguinarine for 24 h. Magnification at 20x (scale bar, 100 μm). n, 10-12 each from 3 independent experiments.

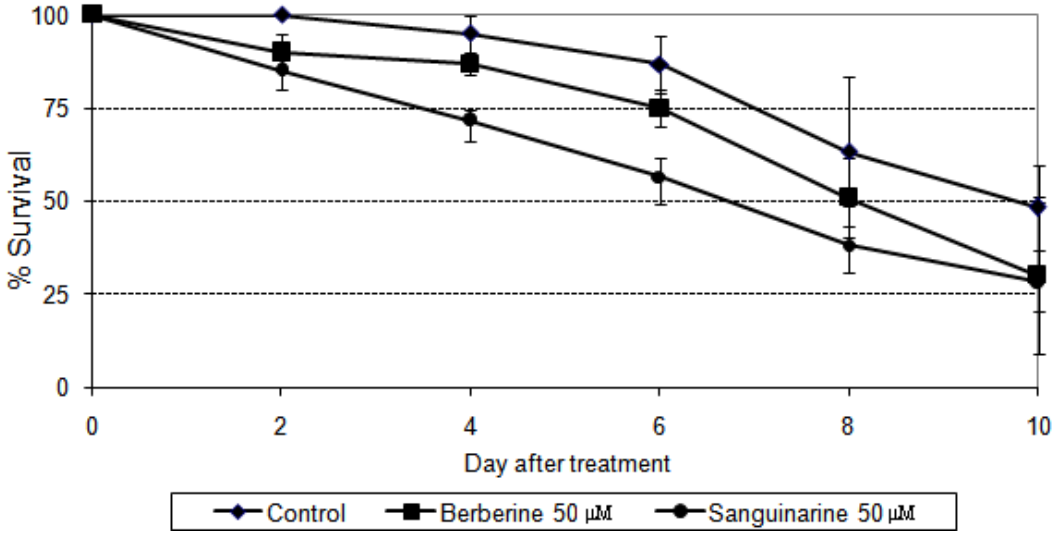


Supplemental Figure 4

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. *$p < 0.05$, **$p < 0.005$, two-tailed *t*-test. *pL4440* – RNAi vector control, *aak-1i* – *aak-1 RNAi worms, aak-2i* – *aak-2* RNAi worms.



Supplemental Figure 5
Oil Red O staining in (A) RNAi vector control, pL4440, and (B) *nhr-49*i worms treated with berberine or sanguinarine for 24 h. Magnification at 20x (scale bar, 100 μm). n, 10-12 each from 3 independent experiments.



Supplemental Figure 6
Survival rate *of C. elegans* after treatment with berberine or sanguinarine monitored until 50% mortality. Results shown as average of two independent experiments. Error bar indicates standard deviation; n, 120.

Supplemental Methods

RNA Interference. 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 1mM isopropyl-b-D-thiogalactopyranoside (IPTG) for the final 3 h. 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 d, during which period the worms would have laid eggs that then hatched and grew to the L3 larval stage. Two-d-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 h 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.

Immunoblot Analysis. 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) 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) and probed with phospho-Thr172-AMPK [1/2000x dilution in 5% BSA/Tris buffered saline with Tween-20 (TBST)], followed by HRP-conjugatedsecondary donkey anti-rabbit IgG (1/5000x dilution in 5%BSA/TBST) and chemiluminescence was detected using ImageQuant LAS4010 (GE Healthcare Life Sciences). Blots were then stripped and reprobed with AMPK (1/2000x dilution in 5%BSA/TBST) and subjected to chemiluminescence detection as with phospho-specific antibody.

Survival Rate. Twenty adult nematodes were placed onto each 6-cm NGM medium plated with *E. coli* OP50 as food source and alkaloids at the indicated concentration for each plate. Survival rate monitoring commenced on "Day 0 after treatment" until 50% of the worm population in each treatment were dead. Live worms were counted and transferred to new NGM + OP50 plates with same alkaloid concentration every 2 d. Worms that did not respond to light touch with a platinum wire picker were considered dead.