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Using *Caenorhabditis elegans* as a Model for Obesity Pharmacology Development

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Abstract

The *Caenorhabditis elegans* model is a rapid and inexpensive method to address pharmacologic questions. We describe the use of *C. elegans* to explore 2 pharmacologic questions concerning candidate antiobesity drugs and illustrate its potential usefulness in pharmacologic research: (1) to determine a ratio of betahistidine–olanzapine that blocks the olanzapine-induced intestinal fat deposition (IFD) as detected by Nile red staining and (2) to identify the mechanism of action of a pharmaceutical candidate AB-101 that reduces IFD. Olanzapine (53 µg/mL) increased the IFD ($12.1 \pm 0.1\%$, $P < 0.02$), which was blocked by betahistidine (763 µg/mL, $39.3 \pm 0.01\%$, $P < 0.05$) in wild-type *C. elegans* (N2). AB-101 (1.0%) reduced the IFD in N2 ($P < 0.05$), increased the pharyngeal pumping rate ($P < 0.05$), and reversed the elevated IFD induced by protease inhibitors atazanavir and ritonavir ($P < 0.05$). AB-101 did not affect IFD in a ACS null mutant strain *acs-4(ok2872) III/hT2[bli-4(e937) let-?(q782) qIs48](I;III)* suggesting an involvement of the lipid oxidation pathway and an upregulation of CPT-1. Our studies suggest that *C. elegans* may be used as a resource in pharmacologic research. This article is intended to stimulate a greater appreciation of its value in the development of new pharmaceutical interventions.

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Keywords

fat; energy; obesity

INTRODUCTION

Obesity pharmaceutical research to define drug mechanisms of action and develop drug dosing information has been typically performed in rodent animal models. *Caenorhabditis elegans* is a model organism that provides advantages by giving quicker answers at a reduced cost, even if results require confirmation in rodents or other animal species later. We used *C. elegans* to determine the approximate dosing ratio for an antiobesity drug being developed to combat olanzapine-induced weight gain. Rapid metabolism of the candidate antiobesity drug made pharmacokinetic studies impractical, but the constant presence of the drugs in the media with *C. elegans* obviates this problem, which would have been a problem in rodents. We also demonstrated that *C. elegans* can be used to determine the mechanism by which a candidate antiobesity drug might cause weight loss. We describe these studies in the hope that broader use of the *C. elegans* model organism will help to speed the pace and reduce the cost of developing pharmaceuticals for the treatment of obesity and other diseases.

The nematode animal model, *C. elegans*, is easy to genetically modify, and many mutations of specific genes are inexpensively available.¹ The *C. elegans* Genetics Center (CGC, UMN) is funded by National Institutes of Health, which maintains and provides a variety of strains. *C. elegans* is easy to maintain with a short lifespan in the laboratory, grows on agar in petri dishes with the standard laboratory food being nontoxogenic *Escherichia coli* (OP50), and favors rapid research results. Approximately, 300 gene inactivations in *C. elegans* have been shown to cause fat reduction, and approximately, 100 gene inactivations cause fat accumulation.² Although *C. elegans* has been used for large-scale, high-throughput screenings to discover drugs and bioactive materials, it has been less commonly used in dose selection or for defining mechanisms of drug action.³

C. elegans deposits fat for energy storage along its intestinal tract. The translucent bodies of the nematodes are convenient for analysis of intestinal fat deposition (IFD) by hydrophobic lipid dye-staining and photometric quantification in the intact animal. Fluorescent probe Nile red has been validated through lipid extraction, separation with thin layer chromatography, and quantification with gas chromatography.^{4,5} Recently, Nile red staining in vitro has been recommended in comparison with coherent anti-Stokes Raman scattering microscopy.^{6,7} *C. elegans* also has rapid reproduction cycles and large brood sizes that allow for the performance of a variety of cellular, molecular, genetic, and behavioral analyses.⁸ The pharyngeal pumping rate (PPR) in *C. elegans* is correlated to the lifespan and is a well-accepted surrogate marker for studies of aging.⁹ The biosynthetic and metabolic pathways of *C. elegans* are highly conserved and are shared with mammalian systems. More than 65% of the genes relating to human diseases are preserved in *C. elegans*,^{1,10–12} approximately, 3500 genetically manipulated strains are marvelous models for unraveling human diseases,^{13–16}

and the *C. elegans* model organisms are helpful to answer pharmacological questions of human diseases.

Question I

Betahistidine and olanzapine-induced IFD—Betahistidine was approved in the United States for the treatment of vertigo in 1968 when safety was the only criteria for approval by the US Food and Drug Administration (NDA 14–241). It was taken off the market in the United States only in 1972 because required efficacy studies were never done (http://money.cnn.com/2007/08/09/magazines/business2/obesity_pill.biz2/index.htm). Betahistidine is currently used throughout the rest of the world and the manufacturer estimates that 60 million individuals have used the drug with minimal and tolerable side effects [Serc (Betahistidine Dihydrochloride), Product Monograph; Solvay Pharma, India, Ltd. Date of revision: September 23, 2003, control #075422]. The adverse drug reaction information system of British Medicine Control Agency lists 299 reports for a total of 461 adverse reactions to betahistidine. The most common adverse reactions are nausea ($n = 19$), vomiting,¹⁷ diarrhea,¹⁸ headache,¹⁹ and pruritis.¹⁷ Not only does betahistidine have a good record of safety but a study in guinea pigs suggests it may also be effective for the treatment of vertigo.²⁰

Betahistidine, a histamine-1 receptor (H_{1R}) agonist and a partial antagonist of the presynaptic autoreceptors of the histamine-3 receptor (H_{3R}), is an orally administered drug that affects central nervous system function by acting on 2 G protein-coupled metabotropic receptors. The H_{1R} seems to control feeding and body weight in animals¹⁸ by reducing food intake mediated through H_{1R} ²¹ and increasing feeding through H_{1R} antagonism in rodents.²² Overwhelming weight gain characterizes the second-generation antipsychotics (SGA), which antagonize H_{1R} , as occurs with olanzapine, whereas betahistidine has been effective in combating olanzapine-induced weight gain.²³ Betahistidine may reverse the effect of H_{3R} antagonists by uncoupling the G protein-coupled reactions to release the H_{1R} from inhibition by the H_{3R} .¹⁷ In patients, taking 12.5–17.7 mg of olanzapine per day, the average weight gain was 12 kg at 1 year.²³ Finding optimal doses of betahistidine may reduce the adverse effect of weight gain by SGA during successful treatment of psychiatric disorders. Betahistidine, however, is metabolized rapidly making it difficult to measure blood levels of the active compound and determine the optimal ratio.²⁴ In *C. elegans*, olanzapine has been shown to increase IFD by 15% in 48 hours.²⁵ The increased IFD may provide an opportunity to define the optimal ratio of betahistidine to olanzapine and to block the olanzapine-induced weight gain. Using *C. elegans* also gets around the problem of rapid betahistidine metabolism, since betahistidine is added to the solution from which *C. elegans* constantly consumes in the culture dish. Although *C. elegans* does not have a histamine receptor per se, it has octopamine receptors,²⁶ whereas octopamine is also a beta(3)-adrenergic agonist in humans.^{27,28} Octopamine receptors have been demonstrated to selectively bind epinastine, a H_{1R} antagonist in humans, suggesting that the octopamine is a homolog of the human H_{1R} and cause moderate weight loss in rodents.^{29,30} In view of these findings and the response to olanzapine with rapid fat gain, we felt that *C. elegans* was an appropriate model to explore an optimal ratio of betahistidine to olanzapine for blocking fat gain.

Question II

Mechanism of action of antiobesity drug candidate AB-101—AB-101 is as successful as metformin, verapamil, or sibutramine, in treating diabetes, hypertension, or obesity, respectively, in rodents (unpublished data). These effects were associated with increased fat oxidation, elevated metabolic rate, and reduced serum free fatty acids. The mechanism of action of AB-101, however, is not known. Metabolism of long-chain fatty acids requires several crucial pathways: (1) intracellular transport of the membrane fatty acid transporter cluster differentiation transport protein (CD36), where CD36 is an upstream regulator of CPT-1 in transporting fatty acids, (2) acetyl-CoA synthetase (ACS) enzymatically binds fatty acids to acyl CoA to form acetyl-CoA, (3) the fatty acid must be bound to carnitine through the enzymes carnitine palmitoyltransferases (CPT-1 and CPT-2) to move across the 2 mitochondrial membranes and enter the tricarboxylic acid cycle or electron transport chain for energy production, and (4) malonyl CoA that is synthesized by acetyl coenzyme A carboxylase (ACC) and downregulates CPT-1 based on the need for lipid synthesis.

CPT-1, ACC, and ACS are also essential elements in the fat oxidation pathway in *C. elegans*. The key enzyme CPT-1 is not able to stimulate fat oxidation in the absence of ACS or CD36, and CPT-1 cannot be downregulated in the absence of ACC. Protease inhibitors atazanavir and ritonavir have been shown to inhibit CPT-1 and CD36 and to increase fat deposition in wild-type *C. elegans*.³¹ We hypothesized that AB-101-induced body fat reduction required mechanisms that involved upregulation of CPT-1, then AB-101 should reduce fat deposition by removing the proteinase inhibitor-induced inhibitory effect on CPT-1 and CD36. Likewise, the AB-101-induced body fat reduction would be absent in the ACS null mutant.

The present report describes 2 proof-of-concept studies using *C. elegans* to answer pharmacological questions: (1) to determine an optimal ratio of betahistine to olanzapine required to minimize olanzapine-induced IFD using wild-type *C. elegans* and (2) using inhibitors of CPT-1 and the ACS null mutant to explore the mechanism of action of AB-101.

MATERIALS AND METHODS

All strains of *C. elegans* and a standard laboratory food source of *E. coli* (OP50) were obtained from the *Caenorhabditis* Genetics Center (CGC, University of Minnesota, MN). Animals were cultured in an agar dish in a low-temperature incubator at 20°C. A day before the experiment, 200 µL of a feeding media was added to the agar dish, and the animals were allowed to eat ad libitum. Control animals were fed with 200 µL of the *E. coli* only.

C. elegans culture

Age-synchronized *C. elegans* were grown on nematode growth media (NGM) agar plates (35-mm diameter) in a 20°C incubator (Revco Tech., Nashville, NC). Nematode growth media culture contained 3 g of NaCl, 20 g of Bacto-agar, 2.5 g of Bacto-peptone (Becton, Sparks, MD), 0.005 mL of 0.1% cholesterol solution in 95% ethanol, and 975 mL of dH₂O, with further addition of 1 mL of 1 M CaCl₂, 1 mL of 1 M MgSO₄, and 25 mL of 1 M KPO₄, pH 6 after autoclaving. Cultures were fed ad libitum with Luria Bertani broth (200 µL)

containing 5×10^8 to 5×10^{11} CFU/mL *E. coli* OP50.³² Mature, gravid *C. elegans* were individually transferred onto agar plates and treated with a 1:1 solution of 0.4 M NaCl and 1 M NaOH to dissolve the *C. elegans* and release the viable eggs. All experimental treatments were conducted 3 days after hatching. Age synchronization was achieved by transferring worms every other day. Two hundred microliters of each treatment was added to fresh agar plates before the transfer. Each group of worms was collected and fixed at 15–20 days as previously described.³³

Study 1—Wild-type *C. elegans* (N2) were divided into 3 groups: group 1 served as an untreated control and received OP50 only; group 2 was divided into 2 subgroups treated with (1) olanzapine (53 µg/mL) or (2) betahistine (763 µg/mL) based on the doses of atypical antipsychotic medication in humans; and group 3 was treated with both olanzapine (53 µg/mL) and betahistine (763 µg/mL).

Study 2—*C. elegans* N2 or *acs-4(ok2872) III/hT2[bli-4(e937) let-? (q782) qIs48](I;III)* (ACS mutant) were used. Group 1 was the control that received OP50 only. Group 2, 3, and 4 were further subdivided and received additional treatments. Group 2 was fed AB-101: (1) 0.3% and (2) 1%. Group 3 received the protease inhibitors atazanavir plus ritonavir at the following combined doses: (1) 3.3 µmole/L/0.83 µmole/L, (2) 6.7 µmole/L/2.5 µmole/L, (3) 20 µmole/L/5 µmole/L, and (4) 33 µmole/L/8.3 µmole/L (Table 1). Group 4 received AB-101 (1%) plus the 3 combinations of the protease inhibitors as in group 3a, b, and c.

At the end of the experiments, an S-basal (see below) solution (1 mL/dish) was used to wash the animals. The solution containing the animals was centrifuged for 20 seconds at 3000 rpm, and this procedure was repeated twice. Animals were then fixed with 4% paraformaldehyde for over 2 hours at 4°C and washed twice with PBS for 5 minutes. The lipid staining dye Nile red was selected to determine the IFD response to the treatments. Nile red was dissolved in acetone. Glycerol–water (75:25) was added into the stock solution. Fifty microliters of the working solution was applied to the animals for 10 minutes. A drop of Fluoromount-G was applied to a glass slide followed by 20 µL of the medium containing Nile red stained animals. The slides were viewed with a fluorescence microscope (Nikon Eclipse, Ti) equipped with a TRITC filter. Digital images were taken with Retiga 4000R and fluorescence micrographs were taken. All data were analyzed offline. The line profiles of the optical densities of the Nile red staining were determined using ImagePro Plus. Unit optical densities (arbitrary units, fluorescent intensity/area) of Nile red labeled IFD were determined for each group at the conclusion of the studies and were compared with their paired control groups.

Statistics were performed using SAS (Version 9.3, SAS Institute Inc., Cary, NC). Data were compared using the Student *t* test for IFD or analysis of covariance (ANCOVA) for PPR. Statistical significance was set at *P* = 0.05.

RESULTS

The first study proved that betahistine inhibited the olanzapine-induced increased IFD in *C. elegans*. The second study confirmed that AB-101 decreased fat deposition in N2, also

reduced the increased fat deposition that was induced by protease inhibitors in N2, and had no effect on the low IFD of the ACS mutant.

Study 1

The control group displayed normal IFD (Figure 1A). Animals receiving olanzapine (53 µg/mL) alone showed a $12.1 \pm 0.1\%$ increase of fat deposition in *C. elegans* (N2) (Figure 1B, $P < 0.02$). Betahistine (763 µg/mL) alone did not alter the fluorescence intensity of Nile red (Figure 1C, $P > 0.05$) but did significantly block fat accumulation engendered by olanzapine in the wild-type *C. elegans*. Combination of olanzapine (53 µg/mL) with betahistine (763 µg/mL) reduced IFD compared with the olanzapine-treated group by $39.3 \pm 0.01\%$ ($P < 0.05$) (Figure 1D, E).

Study 2

AB-101 (1.0%) significantly reduced the IFD (Figure 2A, $P < 0.05$) without impairing growth in N2 animals (data not shown). Both doses elevated the PPR ($P < 0.05$) (Figure 3A). The Nile red intensity was dramatically lower in *acs-4* mutant (Figure 2B), whereas the pumping rate was elevated in the presence of AB-101 (Figure 3B, $P < 0.05$). Combinations of protease inhibitors atazanavir and ritonavir at the higher dosage (Table 1) showed a tendency to elevate IFD by themselves (Figure 2C, $P > 0.05$), which was reversed in the presence of AB-101 (1.0%) in the N2 animals (Figure 2D, $P < 0.05$). The PPR was reduced with or without AB-101 in the presence of atazanavir and ritonavir in N2 (Figure 3C, D, $P < 0.05$).

DISCUSSION

The 2 studies proved the practical use of *C. elegans* model organism to address pharmacological questions. These data are promising in terms of understanding the mechanism of action(s) required by the FDA for potential new drug products. Betahistine does not affect food intake and appetite in humans whereas reduces body weight in healthy and middle-aged obese women,^{34,35} it did not reduce IFD in this study. The most important observation is the blocking by betahistine of olanzapine-induced fat gain. The first study confirmed that betahistine inhibited the olanzapine-induced IFD in *C. elegans* as has been seen in rats and in humans.^{23,36} Olanzapine has been shown to increase fat in *C. elegans*.¹⁹ We demonstrated that the ratio of betahistine to olanzapine used in this study completely inhibited the olanzapine-induced fat gain. This information suggests that the partial inhibition of olanzapine-induced weight gain seen in the human and rat studies may represent an inadequate ratio of the 2 drugs or a consequence of rapid betahistine metabolism. Clearly, more dose–response work is needed going forward to determine the optimal ratio of betahistine to olanzapine capable of maximizing the inhibitory effect on the olanzapine-induced weight gain and a timed-release form of betahistine may also be required. Successful titration of the ratio of the olanzapine/betahistine combination may be extended to titration of betahistine and other SGAs with weight-gaining properties. The *C. elegans* model certainly is cost effective for initial screening to determine the optimal dose–ratio when compared with studies performed in rodents, which incur greater cost and necessitate a longer time to determine the correct answer. An additional advantage to *C.*

C. elegans is that they are not regulated by animal care and use committees, making their use much more time efficient from a paperwork perspective. Betahistine is very rapidly metabolized so that measuring serum levels is impractical. By using the *C. elegans* model to explore the optimal dose ratio of the drug combination, a more focused study to confirm this ratio can be done in rodents and later in human clinical trials. Whether a time-released dose of betahistine will be eventually needed in humans for optimal efficacy due to the rapid metabolism remains to be determined.

In the second study, using the ACS null mutant strain, we investigated a mechanism of action of a new antiobesity candidate drug AB-101 that reduces IFD in the *C. elegans* model by upregulating CPT-1. Treating the ACS mutant with AB-101 was ineffective suggesting that the target of the drug was indeed in the CPT-1 fat oxidation pathway, which includes ACS. Proteinase inhibitors that decrease fat oxidation by inhibiting CPT-1 and CD-36 did increase fat accumulation in *C. elegans*. In addition, in the presence of proteinase inhibitors, the fat accumulation was reversed by AB-101 treatment in *C. elegans*. These data confirmed that AB-101 was working to decrease IFD by acting on the fat oxidation mediated by an upregulation of CPT-1 because it reversed the reduction of CPT-1 activity induced by the protease inhibitors. This study calls for additional work to determine whether the upregulation of CPT-1 is by direct stimulation of the enzyme or, more likely, by inhibition of ACC, which controls the synthesis of malonyl CoA, an inhibitor of CPT-1.

C. elegans model is evolutionarily distant from higher species. Despite its convenience and cost effectiveness, confirmatory studies will still be needed in higher animal model vertebrates. The fact that about 1/3 genes involved in human diseases are not represented in *C. elegans* presents limitations to the study of some human diseases and caution should be exercised in studying those human diseases. We suggest that using *C. elegans* model should be considered for initial proof-of-concept studies that are viewed as screening studies, which will require verification in vertebrate models before human studies.

CONCLUSIONS

These 2 studies in *C. elegans* emphasize the importance of this animal model, as an initial screening step, for accelerating drug development in a cost-effective manner. The initial use of *C. elegans* may offer a faster and more cost-effective path for new pharmaceutical candidate drug(s) to reach the market and benefit people suffering from the targeted diseases. Although the results of studies in *C. elegans* will often need confirming in rodent models, the use of *C. elegans* will allow those rodent studies to be more focused, take less time, and incur a lower cost. We feel that *C. elegans* may represent an under-used tool for pharmacological studies and hope this article will stimulate a greater appreciation of its value in the development of new pharmacologic interventions.

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FIGURE 1.

Fluorescent intensity of Nile red representing IFD. (A) Control, (B) olanzapine (53 µg) increased the fluorescent intensity, (C) betahistine (763 µg) did not increase the intensity, and (D) the olanzapine-elevated fluorescent intensity was reduced in the presence of betahistine. (E) Quantification of the Nile red fluorescence intensity of the IFD. Statistical significance ($P < 0.05$) is illustrated between columns labeled with 1 or between groups labeled with 2. * $P < 0.05$.

FIGURE 2.

AB-101 (1.0%) reduced IFD in N2 but not the *acs* mutant. (A) AB-101 reduced the fluorescence intensity in N2 (●, $P < 0.05$). Low fluorescent intensity of Neil red was detected in *acs-4(ok2872) III/hT2[bli-4(e937) let-?(q782) qIs48](I;III)* with or without AB-101 (○). (B) Combination of atazanavir and ritonavir had a tendency to increase the fluorescent intensity (○). The elevation of the fluorescent intensity in the presence of the combination of atazanavir and ritonavir was reduced by AB-101 (●, $P < 0.05$). * $P < 0.05$ compared with control.

FIGURE 3.

AB-101 (1.0%) elevated the PPR in N2 and ACS mutant but not in the presence of protease inhibitors. (A) AB-101 increased the pumping rate in N2 ($P < 0.05$). (B) AB-101 increased the pumping rate in *acs-4(ok2872) III/hT2[bli-4(e937) let-?(q782) qIs48](I;III)*. (C) Combination of atazanavir and ritonavir reduced the pumping rate in N2 ($P < 0.05$). (D) AB-101 did not reverse the reduced pumping rate by combination of atazanavir and ritonavir.

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Table 1

Doses or protease inhibitors.

Treatment	Doses (μmole/L)				
Atazanavir	0	3.3	6.7	20	33
Ritonavir	0	0.8	2.5	5.0	8.3