Evolutionary conservation of drug action on lipoprotein metabolism-related targets[S]

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Abstract **Genetic analysis has shown that the slower than normal rhythmic defecation behavior of the *clk-1* mutants of *Caenorhabditis elegans* is the result of altered lipoprotein metabolism. We show here that this phenotype can be suppressed by drugs that affect lipoprotein metabolism, including drugs that affect HMG-CoA reductase activity, reverse cholesterol transport, or HDL levels. These pharmacological effects are highly specific, as these drugs affect defecation only in *clk-1* mutants and not in the wild-type and do not affect other behaviors of the mutants. Furthermore, drugs that affect processes not directly related to lipid metabolism show no or minimal activity. Based on these findings, we carried out a compound screen that identified 190 novel molecules that are active on *clk-1* mutants, 15 of which also specifically decrease the secretion of apolipoprotein B (apoB) from HepG2 hepatoma cells. The other 175 compounds are potentially active on lipid-related processes that cannot be targeted in cell culture. One compound, CHGN005, was tested and found to be active at reducing apoB secretion in intestinal Caco-2 cells as well as in HepG2 cells. This compound was also tested in a mouse model of dyslipidemia and found to decrease plasma cholesterol and triglyceride levels.** Thus, target processes for pharmacological intervention on lipoprotein synthesis, transport, and metabolism are conserved between nematodes and vertebrates, which allows the use of *C. elegans* for drug discovery.—Hihi, A. K., M-C. Beauchamp, R. Branicky, A. Desjardins, I. Casanova, M-P. Guimond, M. Carroll, M. Ethier, I. Kianicka, K. McBride, and S. Hekimi. **Evolutionary conservation of drug action on lipoprotein metabolism-related targets.** ***J. Lipid Res.*** **2008.** 49: **74–83.**

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The nematode *Caenorhabditis elegans* is an invaluable model for many aspects of biomedical research, including pharmacology (1, 2), and could be particularly useful for identifying and studying lipid-lowering molecules. Indeed, it is well established that nematodes are auxotrophic for sterols. Although they possess a number of enzymes that can modify sterols (3, 4), they are incapable of de novo sterol biosynthesis; therefore, they depend upon exogenous sterol for their growth and reproduction (5). They exhibit numerous phenotypes under conditions of cholesterol deprivation (4, 6, 7); therefore, perturbations that decrease their internal sterol levels can easily be detected. Furthermore, a recent RNA interference screen for genes involved in fat storage identified a number of genes with clear mammalian homologs that are known to be involved in lipid metabolism (8, 9), and *ncr-1* and *ncr-2*, the worm homologs of the gene responsible for Niemann-Pick type C1 disease, appear to be involved in intracellular cholesterol processing, as is the human Niemann-Pick type C1 gene (10, 11).

We recently discovered a new way to study lipid metabolism in *C. elegans* using the *clk-1* mutants of *C. elegans*. *clk-1* encodes a mitochondrial protein that is necessary for ubiquinone biosynthesis (12) but also has other mitochondrial functions (13). *clk-1* mutants display a variety of developmental and behavioral phenotypes (14, 15), including an average slowing of several rhythmic behaviors, such as pharyngeal pumping, which serves to ingest bacteria, and the defecation cycle. Defecation consists of three distinct muscle contractions that are needed to expulse gut contents through the anus and that are executed within 5 s. This series of three muscle contractions is repeated every 50–60 s, which constitutes the defecation cycle (16, 17). In *clk-1* mutants, the defecation cycle is more typically ~85 s (13). We have found that the slow cycle of *clk-1* could be suppressed by a mutation in *dsc-4* (13), which encodes the worm homolog of the microsomal tryglyceride transfer protein (MTP) (18), a protein whose activity is crucial for the formation and secretion of

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apolipoprotein B (apoB)-containing lipoproteins in mammals (19). Loss of the function of *dsc-4* also suppresses a developmental phenotype of *clk-1*, its slow germline development (18). We have shown that the suppression of the slow germline development by *dsc-4* can also be mimicked by decreasing media cholesterol levels or by using RNA interference to knock down the worm homologs of genes involved in lipoprotein structure, such a vitellogenins (18). This has provided new evolutionary and functional relationships among large lipid transfer protein family members, such as MTP, apoB, and the vitellogenins (20, 21). Furthermore, these findings suggest that the mechanisms of lipoprotein secretion are indeed significantly conserved in worms and are altered in *clk-1* mutants.

Here, we show that the slow defecation, but not the slow pumping, of *clk-1* can be suppressed by low dietary cholesterol as well as by drugs that are known to affect lipid metabolism in vertebrates, but not by drugs that affect processes not directly related to lipid metabolism. This indicates further that altered lipoprotein metabolism is indeed the main cause of the slow *clk-1* defecation cycle. The length of the defecation cycle can readily be scored in a quantitative manner. Thus, we reasoned that a pharmacological suppression of the slow defecation cycle of *clk-1* mutants could be used to identify compounds that affect lipoprotein metabolism in vertebrates as well. We have carried out such a screen and identified a collection of active compounds, including compounds that act on apoB secretion in cultured human cells and in a mouse model of dyslipidemia.

MATERIALS AND METHODS

Chemicals and small-molecule library

We used the following chemicals: fluvastatin, lovastatin, gemfibrozil, probucol, ethosuximide, naphthalene, trimethadione, LiCl, and Triton WR1339 (Sigma-Aldrich, St. Louis, MO) as well as ciglitazone and T0901317 (Cayman Chemicals). The HDL-increasing molecule (*S*)-5-chloro-2-methylphenylthiocarbamoyl-2-ethylpiperidine (referred here as CS-9) was synthesized by Naeja, Inc. (Edmonton, Alberta, Canada). A structurally diverse small-molecule library of 20,000 compounds was obtained from Tripos, Inc. (St. Louis, MO). The library was kept frozen at −20°C in DMSO (10 mM stock).

Worms

Worm strains were maintained at 20°C using standard culture methods. The wild-type strain used was the *C. elegans* Bristol strain N2. The mutants used were *clk-1(qm30)* and *clk-1(qm30);dsc-4(qm182)*. To test drugs, we used small 3.5 cm diameter plates containing solid nematode growth medium-agar medium supplemented with 2 mg/l cholesterol, or as indicated. Compounds were dissolved in the appropriate solvent and spread onto plates. The final concentrations ranged from 20 to 150 μM, depending on the compound. A total of 50 μl of the *Escherichia coli* OP50 strain was then added as a food source, and the plates were incubated overnight at 37°C. Ten to 15 *clk-1(qm30)* or wild-type L1 larvae were then transferred to the test plates and grown at 20°C. The effects of the statins were scored after raising the worms on the compounds for two generations. Defecation cycle rates were evaluated in young adult worms, as described previously (13).

For the analysis of the compounds and for the effects of cholesterol concentration, worms were scored for three consecutive cycles and the defecation rate of each worm was derived from the average. Wild-type worms were also exposed to known lipid-lowering molecules, without a statically significant effect on the defecation cycle. We tested lovastatin (53.3 ± 3.3 s vs. 53.3 ± 2.6 s for controls), fluvastatin (54.6 ± 5.0 s vs. 53.6 ± 4.7 s for controls), gemfibrozil (56.4 ± 4.4 s vs. 58.1 ± 4.3 s for controls), ciglitazone (56.5 ± 4.2 s vs. 58.1 ± 3.9 s for controls), T0901317 (54.0 ± 2.5 s vs. 55.3 ± 3.8 s for controls), and CS-9 (59.0 ± 5.8 s vs. 59.4 ± 3.6 s for controls). For the small-molecule library screening, we used 96-well plates containing solid NGM-agar medium supplemented with 2 mg/l cholesterol. Compounds were spread on top of the medium in groups of five, in a volume of 5 μl, at a final concentration of 50 μM each, and left overnight in the dark at 37°C to allow for drying. Moisture was removed from plate lids to reduce humidity. One microliter of concentrated OP50 bacteria was then added to the test wells, and the plates were incubated overnight at 37°C. Ten to 15 L1 *clk-1(qm30)* larvae were then transferred to the test wells and grown at 20°C. A single defecation cycle of a single animal was measured for each test group.

Groups with cycle lengths of <80 s were scored as positive. All compounds from each positive group were tested individually at a final concentration of 50 μM. We identified 205 positive groups (corresponding to 1,025 compounds), from which 190 individual compounds were later found to be positive. We also evaluated on defecation the activity of compounds known to affect worm phenotypes other than defecation. We assayed LiCl (78.7 ± 9.0 s vs. 75.4 ± 8.5 s for controls), ethosuximide (111.8 ± 15.1 s vs. 97.3 ± 7.5 s for controls), naphthalene (85.8 ± 8.6 s vs. 90.1 ± 9.8 s for controls), probucol (92.6 ± 11.1 s vs. 86.2 ± 9.9 s for controls), and trimethadione (108.9 ± 14.6 s vs. 95.5 ± 11.4 s for controls). Note the variation in the values of the controls, which is the result of the use of different solvent systems for each drug. Only naphthalene had a statistically significant effect, at $P < 0.05$. Concentrations were selected that were known to be bioactive (0.1 mM for naphthalene, 0.8 mM for probucol, 10 mM for LiCl, and 14 mM for ethosuximide and trimethadione).

We also tested our collection of lipid-modulating drugs on *clk-1(qm30)* pharyngeal pumping. The methods for exposing worms to compounds were similar to the protocol for defecation above. Pumping rates were measured in 20 young adult worms for three intervals of 30 s. We assayed lovastatin (162.7 ± 12.8 pumps/min vs. 183.5 ± 8.0 pumps/min for controls), fluvastatin (154.7 ± 8.0 pumps/min vs. 175.2 ± 15.2 pumps/min for controls), T0901317 (163.2 ± 10.5 pumps/min vs. 165.3 ± 11.9 pumps/min for controls), gemfibrozil (173.5 ± 14 pumps/min vs. 168.3 ± 10.9 pumps/min for controls), CS-9 (169.8 ± 13.3 pumps/min vs. 167.5 ± 12.2 pumps/min for controls), and ciglitazone (171.8 ± 11.5 pumps/min vs. 168.3 ± 10.9 pumps/min for controls). Thus, none of these compounds suppressed the *clk-1(qm30)* slow pumping rate. Consistently, the *clk-1(qm30)* pumping rate was not changed whether cholesterol was added to plates or not (167.1 ± 11.6 pumps/min vs. 169.7 ± 11.7 pumps/min, respectively; n = 60) and was not accelerated in *clk-1(qm30);dsc-4(qm182)* double mutants (142.9 ± 11.8 pumps/min vs. 173.0 ± 8.3 pumps/min for *clk-1(qm30)*; n = 35).

Cell culture and in vitro assays

We used the human hepatoblastoma cell line HepG2 (American Type Culture Collection No. HB-8065) as a model for lipoprotein secretion. The cells were grown in minimal

essential medium supplemented with 10% FBS, 1% penicillin/streptomycin, 1% sodium pyruvate, and 1% nonessential amino acids. The cells were plated at a density of 0.5×10^6 cells/well on six-well plates and grown for 2 days at 37°C, 5% CO_2. The cells were washed with PBS (1×) and serum-starved overnight before a 24 h treatment with any compound or solvent as control. We also used the intestinal human cell line Caco-2 (American Type Culture Collection No. HTB-37). The cells were grown in minimal essential medium supplemented with 10% FBS, 1% penicillin/streptomycin, 1% sodium pyruvate, and 1% nonessential amino acids. The cells were plated on six-well plates at a density of 0.5×10^6 cells/well and grown for 10 days at 37°C, 5% CO_2. Caco-2 cells were treated with the test compounds, or the corresponding control solvent, for 24 h in serum-free medium.

ApoB-100, apoB-48, and apoA-I secretion levels were evaluated from medium of treated cells using ELISA (ALerCHEK, Portland, ME), according to the manufacturer's instructions. We also evaluated secreted albumin levels using an ELISA (Bethyl Laboratories, Inc., Montgomery, TX). We evaluated MTP activity from treated cells using a system from Chylos, Inc. (New York, NY). Cellular protein concentration was estimated using the Bradford system (Bio-Rad). CHGN005 was also tested in in vitro assays for ACAT activity and HMG-CoA activity modulation at an outsourced site (MDS Pharma Services, Bothell, WA) according to standard procedures.

Mouse studies

CD1 male mice were used in all animal studies. The studies were conducted according to procedures compliant with Canadian Council on Animal Care guidelines. The protocols were approved by the local Ethics Committee of Mispro Biotech Services, Inc. (Montreal, Quebec, Canada). We used Triton-treated mice as a model for induced hyperlipidemia. Animals were starved overnight for pretreatment serum collection. Animals were then treated and deprived of food until the first 4 h bleed. Food was then given overnight and removed again at least 2 h before the terminal 24 h bleed. A 10% water solution of glucose was provided to mice ad libitum at all times. Animals were dosed with an intravenous injection of Triton (500 mg/kg), followed 1 h later by intravenous injection of CHGN005 formulated in a 30% cyclodextrine solution, at a dose of 50 mg/kg. Control groups were dosed with an intravenous injection of the cyclodextrine vehicle alone. Serum samples obtained at 0, 4, and 24 h were sent to Cirion, Inc. (Laval, Quebec, Canada) for lipid profile analysis. Serum levels of total cholesterol and triglycerides were measured according to standard procedures.

Statistical analysis

For evaluation of the effects of hypolipidemic compounds on worm defecation, on secretion in HepG2 and Caco-2 cells, and on plasma levels of cholesterol and triglycerides in mice, we used a two-tailed *t*-test, assuming unequal variance when the sample sizes were too small for accurate assessment of the variance. Analyses were performed using GraphPad Prism version 4.03.

RESULTS

A platform for identifying compounds that affect lipoprotein metabolism

We previously established that decreasing the levels of cholesterol in the growth medium suppresses the slow germline development of *clk-1* mutants (18). We now show that the slow defecation of *clk-1(qm30)* mutants is also suppressed by decreasing medium cholesterol levels (**Fig. 1A**), whereas wild-type worms remain unaffected (Fig. 1B). This sensitivity of the slow defecation phenotype of *clk-1* mutants to variations in cholesterol, and to a reduction of MTP activity (18), suggested that the phenotype might also be sensitive to pharmacological modulators of lipid metabolism.

We tested a range of compounds that affect representative pathways affecting lipoprotein metabolism, including cholesterol synthesis using the HMG-CoA inhibitors fluvastatin and lovastatin, reverse cholesterol transport activation using the liver X receptor (LXR) agonist T0901317 (22), the peroxisome proliferator-activated receptor

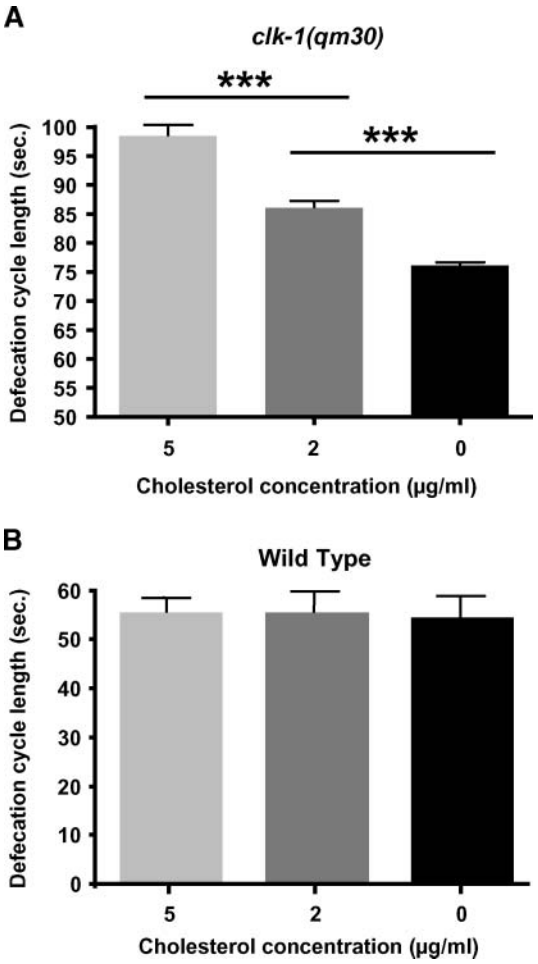

Fig. 1. The defecation cycle length of *clk-1(qm30)* mutants is cholesterol-sensitive. A: Low medium cholesterol partially suppresses the slow defecation period of *clk-1(qm30)* mutants. First, larval stage (L1) animals grown at 20°C in the presence of 5 mg/l cholesterol were transferred to plates containing 5 mg/l (n = 80), 2 mg/l (n = 102), or no added (n = 128) medium cholesterol. In the latter, trace amounts of cholesterol are likely still present from other components of the growth medium. These results show that decreasing the amount of cholesterol in the growth medium accelerates the rate of defecation of *clk-1(qm30)* worms. *** $P < 0.0001$. B: Unlike mutations in *clk-1*, low medium cholesterol does not accelerate defecation in the wild type (N2). The sample sizes for 5, 2, and 0 mg/l were 50, 72, and 70, respectively. Error bars indicate ± SEM.

(PPAR)-γ agonist ciglitazone (23), and the activation of PPAR-α with gemfibrozil, which induces triglyceride decrease and HDL increase in animal models (24). In addition, we tested a phenylthiourea-containing compound (called CS-9 here) that has PPAR-independent HDL-increasing properties in a rat model of hypercholesterolemia (25). We could not test any of the specific MTP inhibitors that have been developed and tested in vivo, as none of these compounds was commercially available. **Figure 2** shows that all of the compounds tested, with the exception of ciglitazone, are capable of accelerating the rate of defecation of *clk-1* mutants. These findings indicate that the active molecules have targets in worms and that interaction with these targets mimics the effect of a genetic inactivation of MTP or of a reduction of dietary cholesterol. Thus, given that these drugs act on aspects of lipoprotein metabolism in vertebrates, it is likely that they act on worm targets that are homologous to their vertebrate targets.

Interestingly, we found that statins are active, although there is no cholesterol biosynthesis in *C. elegans*. However, as their expected target, HMG-CoA reductase, is present in worms, their effects could be mediated by the reduction in some other mevalonate-dependent process. On the other hand, ciglitazone did not appear to be active in our system. However, at this stage, we cannot distinguish whether ciglitazone was not bioavailable in the system or whether it is not active because there is no appropriate target in worms, no nuclear receptor structurally or functionally close enough to PPAR-γ or to any of the ~270 nuclear receptors identified in worms (26).

The drugs were all tested by spreading them onto the agar medium on which the worms were cultured. The concentrations at which activities of the drugs could be observed were determined experimentally for each compound (ranging from 20 to 150 μM) and were often limited by solubility. Indeed, most of the compounds tested are hydrophobic and tend to precipitate at concentrations greater than those that were used to establish activity. Therefore, for any drug that had no effect, it remains possible that the concentration at which we could assay it was too low to show activity. Furthermore, we do not know how well the drugs are absorbed or at what concentrations they accumulate in the various tissues of the animals, which are likely much lower than the concentration in the medium.

We carried out a number of control studies to establish the specificity of the action of the lipoprotein-active drugs in our system. First, we found that decreasing dietary cholesterol availability or treatment with the drugs that were active on *clk-1* mutants had no effect on wild-type animals (see Materials and Methods). This indicates that the drugs act with much more efficiency when lipid metabolism in the worms is perturbed, as it appears to be in *clk-1* mutants. Second, we tested the effect of the drugs on pharyngeal pumping, a rhythmic behavior that acts in food intake and is slowed in the *clk-1* mutants. We found that the slow pumping of *clk-1* mutants is not suppressed by *dsc-4* or by decreasing cholesterol or by any of the drugs tested (see Materials and Methods). Lastly, we tested five drugs that had been shown by others to be active in worms on unrelated phenotypes and that were commercially available: probucol (27), naphthalene (28), trimethadione (29), ethosuximide (29), and LiCl (30). Only naphthalene showed any effect in suppressing *clk-1* (Fig. 2). This effect

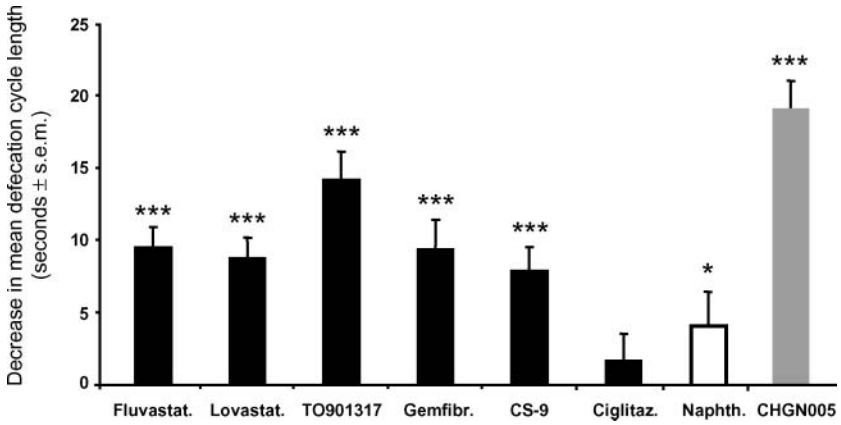

Fig. 2. Hypolipidemic drugs specifically suppress the slow defecation cycle of *clk-1* mutants. The following hypolipidemic molecules were tested: fluvastatin (n = 45), lovastatin (n = 45), the liver X receptor agonist T0901317 (n = 95), gemfibrozil (n = 70), CS-9, which is an HDL-increasing phenylthiourea (n = 81), ciglitazone (n = 56), and CHGN005, which was identified in this study (n = 90). We also tested bioactive drugs that affect other *C. elegans* phenotypes (LiCl, ethosuximide, trimethadione, probucol, and naphthalene). Only naphthalene had a small but significant effect (* $P < 0.05$) and is displayed on the bar graph (n = 45). Maximal concentrations were used. Higher concentrations led either to precipitation of the compound (statins, gemfibrozil, CS-9, and ciglitazone) or to deleterious effects on growth (T0901317). The effects of the drugs are expressed as mean decreases in defecation cycle length ± SEM. All drugs tested had a significant effect (*** $P < 0.001$) except for ciglitazone, which showed no effect. These effects on *clk-1* mutants are similar to those produced by mutation in *dsc-4* [*C. elegans* microsomal tryglyceride transfer protein (MTP)] (13). Error bars indicate ± SEM.

was of borderline significance, and we have no indication at present of the mechanism by which it might act.

In conclusion, our results suggest that molecules able to affect lipoproteins in vertebrates by a variety of mechanisms act specifically on the slow defecation phenotype of *clk-1* mutants.

Use of *C. elegans* to screen a library of low molecular weight compounds

To determine whether the sensitivity of *clk-1* mutants to drugs that affect lipoprotein metabolism could be used to identify new lipoprotein metabolism modulators, we screened a library of 20,000 low molecular weight compounds with a high level of structural diversity. The initial scoring was carried out by measuring a single cycle from a single worm. This approach was used previously in a successful genetic screen for suppressors of the slow defecation phenotype of *clk-1* (13). Also, to speed up the process, compounds were screened in mixes of five compounds in 2.5% DMSO. Compounds were tested at a final concentration of 50 μM. Any compound capable of accelerating the defecation cycle length of *clk-1* worms to <80 s was considered positive. This criterion allows for clear discrimination compared with controls, as could be determined when testing the effects of dietary cholesterol lowering (Fig. 1) and genetic disruption of *dsc-4* (13, 18). We identified 205 positive groups of five compounds that produced a defecation cycle of <80 s. All compounds in these groups (5 × 205 = 1,025) were retested individually in 1% DMSO, yielding 190 individual positive compounds. The groups that did not yield such an individual positive compound might have been false-positives (the single scored cycle was fast by chance alone) or might have produced an effect on the defecation cycle by a combination of effects of more than one compound in the group (**Fig. 3**). The positive compounds represent ~1% of the library, which is an understandably high hit rate given that the screen is expected to hit multiple targets. Beyond yielding candidate compounds for further characterization, the results of the screen also indicated that compounds from a random (but diverse) library only rarely (1:100) affect the length of the defecation cycle; thus, the high hit rate found with lipid-modulating drugs (Fig. 2) is not fortuitous but suggests that these drugs likely act in worms in a manner related to their known vertebrate activities.

Characterization of active molecules on apoB secretion from HepG2 cells

All 190 molecules that were found positive in worms were tested in human hepatocarcinoma cells (HepG2) for their capacity to decrease the secretion of apoB-100 (31) and/or to inhibit MTP (32). ApoB decrease is a recognized surrogate marker for dyslipidemia treatment and is a clinically relevant end point (33). MTP was of interest because of our findings with DSC-4, the *C. elegans* MTP homolog (18). No compound was found to specifically inhibit MTP (data not shown). However, using an ELISA, we found 15 molecules that selectively inhibit apoB secretion at 20 μM (see supplementary Table I). The remainder were either toxic for these cells at that concentration, as they generally inhibited secretion (54 compounds), or had little or no effect on secretion (121 compounds). Thus, we consider that 175 compounds (54 + 121) were negative in the HepG2 screen (Fig. 3).The yield of apoB-decreasing molecules from compounds initially identified in the worm screen was 7.9% (15 of 190). Thus, when considering the two-step screen (acceleration of defecation in *clk-1* mutants and reduction of apoB secretion from human cells), the hit rate for apoB decrease in HepG2 is ~0.1%. As expected from the nature of the screening procedure with worms, in which we have no information about how much of a compound finds its way into the animals, there was no correlation between the efficacy of a compound on

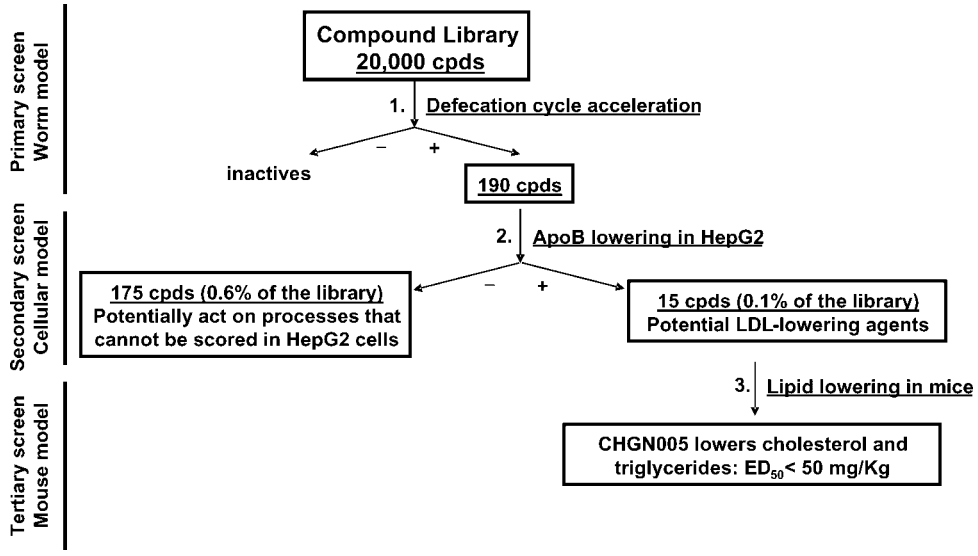

Fig. 3. Flow chart for hypolipidemic small-molecule screening from worms to mice. cpds, compounds.

the defecation cycle and its efficacy in decreasing apoB from HepG2 cells (data not shown). Furthermore, we cannot conclude that all of the compounds that are inactive in worms would necessarily be inactive on lipid metabolism in vertebrates, as there are several possible reasons why they may be inactive in worms, including the absence of the target, too large structural differences in the targets, or a lack of bioavailability in the worms.

Characterization of CHGN005 in human cells and mice

The most active apoB-decreasing compound, CHGN005, was characterized in detail. **Figure 4** shows that CHGN005 reduces apoB secretion from HepG2 cells in a dose-dependent manner, with an IC_{50} of 1 μM, with very little effect on the secretion of apoA-I and albumin or on MTP activity, except at the highest concentrations tested. We also tested CHGN005 in the apoB-48-secreting human intestinal cell line Caco-2 (34), in which CHGN005 also inhibited apoB secretion, albeit less efficiently (see supplementary Fig. I). In addition, we tested the compound in two in vitro biochemical assays: for ACAT activity (35) and HMG-CoA reductase activity (36). The compound did not significantly inhibit either enzyme (data not shown).

An intravenous formulation of 97% pure CHGN005 was administered to mice treated with Triton WR-1339, which induces an acute increase of plasma triglycerides by blocking triglyceride hydrolysis via lipoprotein lipases. Triton treatment also increases the plasma levels of cholesterol and LDL (37). This model has been used successfully in mice and rats for the identification of several lipid metabolism modulators, such as squalene synthase inhibitors (38) and MTP inhibitors (39, 40). **Figure 5** shows that CHGN005, administered at 50 mg/kg by intravenous injection, decreases cholesterol and triglycerides in the Triton model. The effect is most pronounced on cholesterol, for which the ED_{50} at 24 h appears to be <50 mg/kg, whereas triglyceride ED_{50} is ~50 mg/kg. This finding fully validates our approach and our screen, as a compound that was identified based solely on its effect on the defecation behavior in *C. elegans* affects lipid levels in mice. Thus, we expect that many of the other 190 compounds we have identified might also have lipid-lowering activity in vertebrates.

DISCUSSION

Lipoprotein-modulating drug activities are conserved in nematodes

We have used *C. elegans clk-1* mutants, in which lipoprotein and cholesterol metabolism is perturbed, to test the effect of a number of drugs that act on vertebrate lipid metabolism and found that a majority of them indeed affect the phenotype of these mutants but not that of a congenic wild-type strain. These drugs, fluvastatin, lovastatin, gemfibrozil, the LXR agonist T0901317, and the HDL-increasing thiourea CS-9, have virtually no structural similarity and are believed to act on completely different targets. Therefore, our observations suggest that the targets of these molecules are conserved to a sufficient degree in *C. elegans* for these molecules to be active in this organism.

Invertebrate model systems are powerful tools for exploring basic molecular mechanisms and have revealed that molecular structures and processes are highly conserved among living organisms. However, drug discovery has mostly been carried out using vertebrates or vertebrate cells, because a particularly tight relationship between the exact molecular structure of a target protein and the drug that can affect it is expected, and because the action of drugs that must affect the interactions between more than one cell type or organ might be difficult to

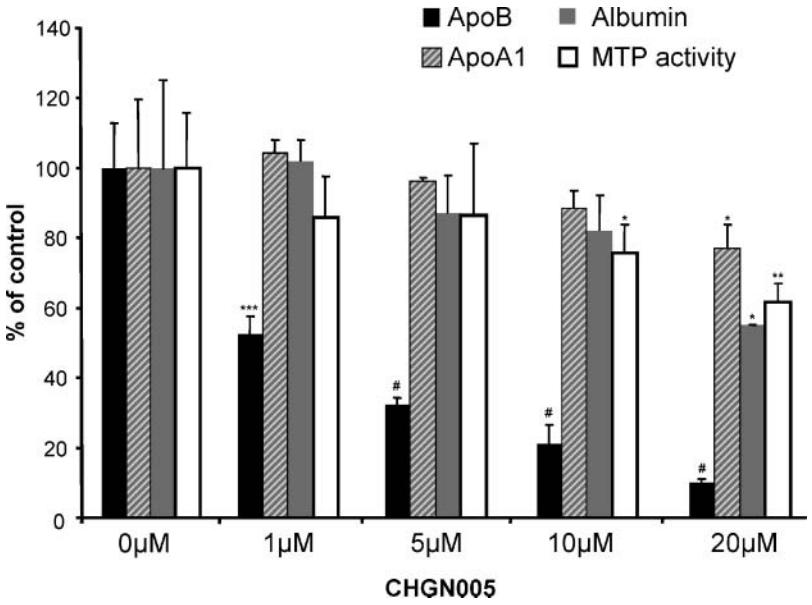

Fig. 4. CHGN005 inhibits apolipoprotein B (apoB) secretion from HepG2 cells in a dose-dependent manner. HepG2 cells were treated with increasing concentrations of CHGN005 for 24 h in serum-deprived medium from 0 to 20 μM. CHGN005 selectively inhibits basal apoB secretion in a dose-dependent manner and significantly affects the secretion of apoA-I and albumin and the activity of MTP only at the highest concentration. Error bars represent SD. All asterisks represent *P* values compared with 0 μM: *** $P = 0.0002$, ** $P < 0.01$, * $P < 0.05$. # $P < 0.02$ compared with the closest lower concentration (given only for apoB). Error bars indicate ± SD.

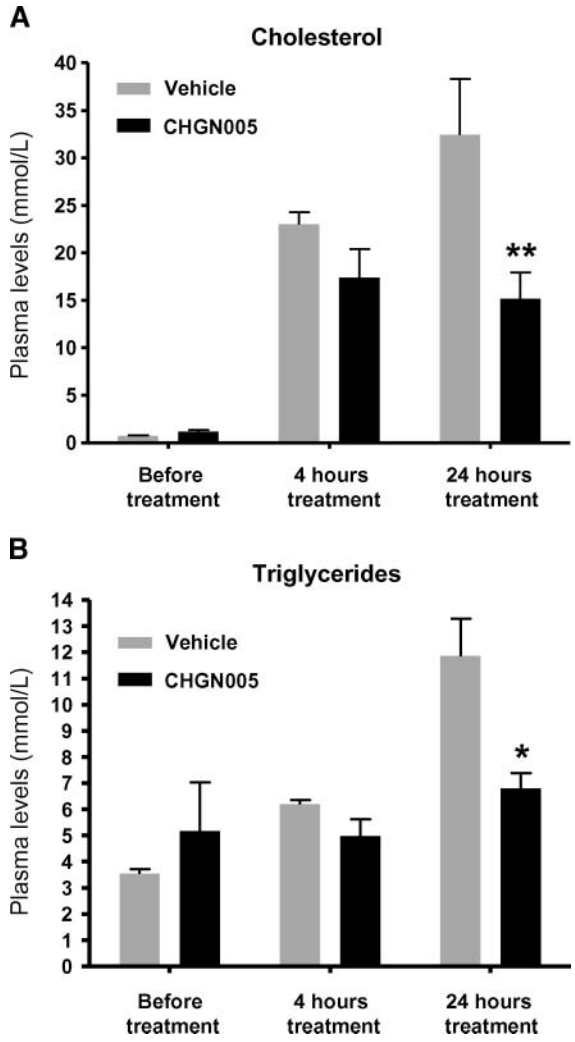

Fig. 5. CHGN005 decreases plasma cholesterol and triglycerides in Triton-treated mice. CD1 male mice (n = 5) were starved overnight for pretreatment serum collection. Animals were then treated and deprived of food until the first 4 h bleed. Food was then given overnight and removed again 2 h before the terminal 24 h bleed. A 10% water solution of glucose was provided to mice ad libitum at all times. Animals were dosed with an intravenous injection of Triton (500 mg/kg), followed 1 h later by intravenous injection of CHGN005, formulated in a 30% cyclodextrine solution, at a dose of 50 mg/kg. Control groups were dosed with an intravenous injection of the cyclodextrine vehicle alone. Error bars indicate SEM. A: Cholesterol levels of CHGN005-treated mice are somewhat lower than those of controls at 4 h after treatment and are significantly different from those of controls after 24 h. ** $P < 0.01$. B: Triglyceride levels of CHGN005-treated mice do not increase significantly after treatment and are significantly different from controls after 24 h. * $P = 0.029$. Error bars indicate ± SEM.

reveal in invertebrates, owing to their simpler and distinct anatomies. For example, lipoprotein metabolism involves intestinal uptake, interactions among circulating lipoproteins, lipid uptake and release by peripheral tissues, uptake by the liver, and the processes of cholesterol synthesis, degradation, and excretion. Yet, we found that the degree of conservation of drug action in lipoprotein metabolism is sufficient to allow for drugs that have been developed in vertebrates to affect *C. elegans*, even in the case of drugs that affect complex processes involving more than one tissue, such as reverse cholesterol transport. The recent finding that lipoprotein secretion can be readily studied in zebrafish (41), a practical vertebrate genetic model, provides an additional possible stepping stone to ultimately discover new drug targets that can be used to alleviate disease in humans.

Inactive drugs

In our system, there are a number of potential reasons for which a drug might fail to show activity. One potential cause of a lack of detectable activity could be the fact that some drugs could not be tested at high concentrations because they were found to precipitate out of solution upon mixing with the agar medium and bacteria on which worms grow. If a drug precipitates without forming large aggregates, this would not be easily observed and the drug would be scored as being inactive. Drugs might also fail to penetrate into the animal, be actively detoxified, or be eliminated from cells. Of course, more specific reasons might also be at work. For example, no worm homolog of the vertebrate target might exist, or the homolog might not be similar enough for the drug to act on it.

A fully validated screening method

By testing known drugs, we have shown that drugs that act in mammals act in worms. This implies that compounds could be identified in worms and then shown to act also in mammals and developed as drugs. We have carried out such a screen and identified 190 compounds, out of 20,000 screened, that suppressed the slow defecation phenotype of *clk-1* mutants. Fifteen of these molecules were then shown to be active in an assay for apoB secretion; the other 175 did not show specific activity. One promising compound was tried in a mouse model of hyperlipidemia and was found to be active at a concentration of 50 mg/kg, which is comparable to the dosage used in this model for some known drugs (38, 42). These findings lead again to the conclusions that in the domain of lipoprotein metabolism, there is extensive overlap between the compound space that is active on *C. elegans* and mammals and that worms can be used to screen efficiently for such compounds. Furthermore, given the genetic tractability of *C. elegans*, the model can be used to identify the molecular target for any active compound.

The multiplicity of possible mechanisms of action

At present, we know that 15 of our compounds are able to decrease the secretion of apoB from human HepG2 cells. However, we do not yet know on which targets these compounds act to achieve this inhibition. However, based on an in vitro assay for MTP activity, we believe that none of the compounds act by specifically inhibiting MTP. Thus, all 15 compounds could act on the same target or on 15 different targets. We investigated one of these compounds (CHGN005) in more

detail and found that it had a specific and dose-dependent apoB-decreasing effect in the micromolar range, both in cultured hepatocytes and in enterocytes. We excluded the possibility that this compound interferes with enzymes that directly or indirectly affect lipoprotein metabolism (MTP, ACAT, HMG-CoA reductase). Thus, at this time, the precise mode of action of CHGN005 remains elusive. However, its activity in two lipoprotein-secreting cell types suggests that it affects an intracellular mechanism shared by both cell types or that a circulating factor is affected that regulates apoB more indirectly. We can also speculate that apoB decrease by CHGN005 is independent from intracellular degradation via ubiquitination, because this regulation mechanism is not well developed in Caco-2 cells (43). Many parameters are known that can affect apoB levels, such as the rate of triglyceride biosynthesis (44), but also mechanisms such as insulin signaling (45), the regulation of LDL receptor transcription (46), and degradation via proprotein convertase subtilisin/kexin type 9 (47). All of these mechanisms could also be relevant in *C. elegans* and thus are potential CHGN005 targets. The multiplicity of mechanisms that are involved in controlling the levels of secreted apoB suggests that a reasonable way to identify the mechanism underlying the action of CHGN005 or any other compound that is active in *C. elegans* might be the use of *C. elegans* molecular genetics, an approach that has been successful in the past (2, 28, 48–52).

In addition to the apoB-decreasing compounds, 175 of 190 compounds found to be positive in worms did not specifically affect apoB secretion. At this stage, we cannot determine whether these compounds just do not affect the mammalian homolog of the worm target on which they act or whether they have hypolipidemic actions distinct from apoB decrease. In fact, the latter possibility is likely, as we have found that gemfibrozil, a phenylthiourea derivative (CS-9), and an LXR agonist are active on *clk-1* mutants, but it is at best unclear whether they could be identified in an in vitro apoB secretion assay (53–58). Interestingly, we actually found CS-9-phenylthiourea-like molecules in our screen (data not shown). Thus, a number of the 175 molecules that are not active on HepG2 cells might act on targets whose action on lipoprotein profiles could only be determined in in vivo animal tests. Furthermore, these could also include molecules that act on previously entirely uncharacterized targets. Of course, it is also possible that some of these molecules do not act on lipid metabolism but affect other pathways involved in the regulation of the defecation cycle (17).

Lipid metabolism in *clk-1* mutants

We previously proposed a model for the mechanism that underlies the ability of mutations in *dsc-4* (the worm homolog of the large subunit of MTP) to suppress some of the effects of *clk-1* mutations (18). In this model, the defecation cycle length is determined by the amount of unoxidized (native) LDL-like lipoprotein particles, with high levels correlating with slow defecation. Our current findings that statins, LXR, and PPARα agonists can have effects on this system suggest two diverging interpretations. These compounds could all act, directly or indirectly, on reducing the amount of native LDL-like particles; however, in the absence of any evidence of HDL-like lipoproteins in *C. elegans*, this is not inconsistent with what is known of their action in vertebrates. On the other hand, there could be a more intimate relationship between *clk-1* and lipoprotein metabolism. Indeed, although currently published results are limited, the expression of *clk-1* (59) and the levels of ubiquinone (60) appear to be under the control of transcription factors, such as LXR and PPARα. Thus, it is possible that the levels of *clk-1* expression and ubiquinone are part of the network of interactions that regulate lipid and lipoprotein metabolism and contribute to reverse cholesterol transport. In this model, the lipid phenotypes that result from a loss of function of *clk-1* can be overcome in part by an overstimulation of the system with activators.

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