THE ANTI-NEURODEGENERATION DRUG CLIQUINOL INHIBITS THE AGING-ASSOCIATED PROTEIN CLK-1*

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Running Head: A Drug that Links Neurodegeneration and Aging

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The development of neurodegenerative diseases such as Alzheimer’s, Parkinson’s, and Huntington’s disease is strongly age-dependent. Discovering drugs that act on the high rate of aging in older individuals could be a means of combating these diseases. Reduction of the activity of the mitochondrial enzyme CLK-1 (also known as COQ7) slows down aging in C. elegans and in mice. Clioquinol is a metal chelator that has beneficial effects in several cellular and animal models of neurodegenerative diseases as well as on Alzheimer’s disease patients. Here we show that clioquinol inhibits the activity of mammalian CLK-1 in cultured cells, an inhibition that can be blocked by iron or cobalt cations, suggesting that chelation is involved in the mechanism of action of clioquinol on CLK-1. We also show that treatment of nematodes and mice with clioquinol mimics a variety of phenotypes produced by mutational reduction of CLK-1 activity in these organisms. These results suggest that the surprising action of clioquinol on several age-dependent neurodegenerative diseases with distinct etiologies might result from a slowing down of the aging process through action of the drug on CLK-1. Our findings support the hypothesis that pharmacologically targeting aging-associated proteins could help relieve age-dependent diseases.

Mutational inactivation of clk-1 in the nematode Caenorhabditis elegans (1), and partial inactivation of its ortho-logue Mclk1 in mice (2), prolong the average and maximum lifespan of these organisms, and slows down the development of biomarkers of aging (3,4). Genetic evidence in nematodes indicates that the mechanism by which clk-1 prolongs lifespan is distinct from that of the insulin signaling pathway but overlaps with that of caloric restriction (5,6). This makes the clk-1/Mclk1-dependent pathway one of the very few molecularly defined and evolutionarily conserved pathways of animal aging that has been shown to be active in mammals (7-9). clk-1/Mclk1 encodes a mitochondrial hydroxylase that is necessary for the biosynthesis of ubiquinone (coenzyme Q or UQ) (10-13). In the absence of CLK-1/MCLK1, both worm and mouse cells accumulate the biosynthetic intermediate de-methoxy-ubiquinone (DMQ). The complete absence of MCLK1 is lethal in mice but homozygous C. elegans clk-1 null mutants survive when dietary UQ is available from their bacterial food source.

There are indications that CLK-1 carries out additional biochemical functions in addition to its role in UQ biosynthesis. The first observations suggesting this came from the molecular and genetic analysis of two alleles of C. elegans clk-1. In particular, it was found that the phenotypically more severe allele clk-1(qm30), a partial deletion that does not produce any CLK-1 protein, and the weaker allele clk-1(e2519), an E to K missense mutation that produces wild-type levels of a full-length mutant protein (14), are equally unable to sustain UQ biosynthesis (13), suggesting that at least part of the phenotype of C. elegans clk-1 mutants is not due to the defect in UQ biosynthesis. Second, it was shown that long-lived Mclk1+/− mutants, which produce only half the amount of wild-type MCLK1 protein (11), display profound changes in mitochondrial function in spite of the presence of wild-type levels of UQ (4). The sensitivity of mitochondrial function to the level of MCL1 expression observed in Mclk1+/− mutants suggests that the second function might not be enzymatic in nature but may involve a stoichiometric relation between CLK-1 and other mitochondrial proteins.
Old \textit{Mclk1}^{-/-} mutants display reduced systemic biomarkers of aging, such as plasma isoprostanes and plasma 8-hydroxydeoxy-guanosine, which suggests that these animals are physiologically younger throughout their lives (3). Furthermore, the reduction of MCLK1 can fully prevent the accelerated deterioration of mitochondrial function and the increased mitochondrial oxidative stress observed in young \textit{Sod2}^{-/-} mutants (3), where loss of one copy of the gene coding for the mitochondrial Mn-superoxide dismutase leads to phenotypes that resemble accelerated aging (15,16).

Clioquinol (5-chloro-7-iodo-quinolin-8-ol) was widely used as a safe intestinal disinfectant until it was banned following suspicion that it might have been involved in an outbreak of subacute myelo-optic neuropathy (SMON) in Japan (17). More recently, possible beneficial effects of the metal chelating properties of clioquinol (CQ) on neurodegenerative diseases and cancer have been explored in a variety of systems (18-23). Surprisingly, CQ appears to be beneficial in animal models of all three major neurodegenerative diseases (AD, PD and HD) (18-20,23), although these are not known to have a common etiology. In addition, preliminary data suggest that CQ has positive effects on AD patients (24-26). A variety of different mechanisms of action have been proposed to account for these effects on disease: copper and zinc chelation (18) or increased level of a metalloprotease (27) for AD, iron chelation for PD (19), inhibition of huntingtin expression from its mRNA and moderation of ROS toxicity for HD (20), as well as zinc (21) and copper (22) transport, and proteasome inhibition when complexed with copper (22,28) as well as stimulation of TNF-\(\alpha\) secretion (29) for cancer.

Genes that modulate lifespan are being studied for a variety of reasons (8). One reason is the possibility that pharmacologically targeting the proteins that they encode could help alleviate age-dependent diseases. Here we show that the drug CQ, which is active on a broad pattern of age-dependent neurodegenerative diseases, is an inhibitor of CLK-1/MCLK1, which is an aging-associated protein.

\textbf{Experimental Procedures}

\textit{Cell culture, drug treatment, and quinone extraction from cells-} Mouse RAW264.7 cells (TIB-71, ATCC) and human Hela cells (CCL-2, ATCC) were cultured under standard conditions. CQ (Sigma) was dissolved in DMSO to a concentration of 10mM, with further dilutions made in culture medium. For preloading cells with metal ions cells were incubated with FeSO$_4$ or CoCl$_2$ in complete medium overnight. Cells were then washed twice before incubation with CQ. Quinones were extracted as described, similarly for bacteria, cells, mouse tissue and worms (12); the elution was monitored by a UV-visible detector at 275nm. Cell protein content was determined with a BCA protein assay kit (Pierce). For all HPLC analyses, UQ$_9$, UQ$_{10}$ and DMQ$_9$ were identified by co-elution with commercial standards or DMQ$_9$ purified from \textit{C. elegans clk-1} mutants.

\textit{Bacterial expression of MCLK1 and UbiFp-\textit{Escherichia coli} strain JF496 (ubiF-)} was obtained from the \textit{E. coli} stock center at Yale University and lysogenized using a \(\lambda\)DE3 kit as recommended by the manufacturer (Novagen). JF496(DE3) bacteria were transformed with pET-based plasmids (Novagen) containing the \textit{Mclk1} (30) or the \textit{E. coli} \textit{ubiF} gene. The latter was amplified from \textit{E. coli} K-12 chromosomal DNA by PCR. Primer sequences are available upon request. For HPLC quinone analysis, the JF496(DE3)-\textit{ubiF} and JF496(DE3)-\textit{Mclk1} strains were diluted from an overnight preculture to an OD$_{600}$ of 0.03 in a 1:1 solution of M9 and LB. Bacterial cultures were incubated for 6 hours at 37°C with agitation in the presence of CQ in DMSO. HPLC analysis was carried out as described (10).

\textit{Nematode culture, drug treatment and HPLC analyses-} CQ dissolved in DMSO was added directly into the standard NGM agar prior to pouring plates. 0.1% tergitol was added to help dissolve the CQ. For experiments in which the phenotype of worms was scored on plates with GD1 bacteria, the worms were always grown first for a full generation on GD1-seeded plates before the generation of the actual experiment. Growth on GD1 and HPLC analyses were carried out as described previously (31). For experiments testing the effect of Fe$^{++}$, an aqueous solution of FeSO$_4$ at a desired concentration was added to the standard nematode growth medium immediately after adding CQ.

\textit{Treatment of mice with CQ-} Three-month-old female C57BL/6 mice (Jackson Laboratory) were randomly assigned to one of the experimental groups. Two different treatments with CQ were
carried out: 24-hour or 5-days. In the 24 hour treatment experiment, mice received two gavages, 18 hours apart, with CQ (40mg/kg in 20% Intralipid (Sigma)) or vehicle alone. In the 5-day experiment, mice received five gavages with CQ or vehicle alone, every 24 hours. For both treatments, mice were fasted for four hours after the last gavage and were killed by cervical dislocation after isoflurane anesthesia, and organs were promptly frozen in the liquid nitrogen.

Western blot analysis of MCLK1- After 24 hours treatment with CQ, cells were lysed in RIPA buffer (50mM Tris-HCl pH 7.4, 150mM NaCl, 1% NP40, 0.25% Na-deoxycholate). Equal amounts of lysates were separated by 12% SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane (Bio-Rad). The membrane was then blotted with the primary antibodies: rabbit anti-MCLK1 (1:1000), mouse anti-porin (1:4000), overnight at 4°C, followed by incubating with the corresponding second antibodies, goat against rabbit IgG, and goat against mouse IgG (Sigma) for 2 hours at room temperature. The bands were visualized by ECL detection (Amersham Pharmacia Biotech).

Analysis of aconitase and UQ from mice- 150-200 mg of liver tissue were processed and fractionated into cytoplasmic and mitochondrial fractions by standard methods. Protein quantification was done using the BioRad assay. Aconitase activity and UQ levels were measured as previously described (2,32).

RESULTS

CQ inhibits the DMQ hydroxylase activity of mammalian CLK-1- To identify small molecules capable of inhibiting the DMQ hydroxylase activity of CLK-1, a compound screen whose results have already been partially described, was carried out (33). One of the molecules that was identified included an 8-hydroxyquinoline moiety, a structure that is the core of CQ. To determine the significance of this resemblance, we assayed CQ on cultured mouse RAW264.7 macrophages and human Hela cells, and found that it inhibited the activity of CLK-1 as evidenced by the appearance of DMQ after 24h of treatment at ≥10µM (Fig. 1A), and in a dose-dependent manner (Fig. 1B). The effect was quickly reversible by removal of the drug (Fig. 1C).

To establish the specificity of the action of CQ on CLK-1 we took advantage of the fact that DMQ hydroxylation, the enzymatic reaction carried out by CLK-1 in eukaryotes (34) and in some bacteria (35), is carried out in E. coli by UbiF, an enzyme with an entirely different structure (36). We used a bacteria strain (JF496) deficient in UbiF function (36), which we complemented with clones carrying either Mclk1 or ubiF, which reconstituted UQ biosynthesis in both cases (Fig. 2, A-C). However, treatment with CQ only affected UQ biosynthesis in the bacteria expressing Mclk1 (Fig. 2, D-F), with the appearance of DMQ further indicating that CQ specifically affects the biosynthetic step that involves CLK-1, rather than UQ biosynthesis more generally.

The inhibition of CLK-1 by CQ is quickly blocked by iron ions- We wondered whether the inhibition of CLK-1 could be related to any of the mechanisms that have been proposed to explain the effect of CQ on neurodegenerative diseases and cancer, and which focus on the chelation of divalent metal cations. To explore this, we treated RAW264.7 cells previously treated with CQ for 24 hours with a variety of ions (Fe++, Cu++, Zn++ and Co++), through a range of concentrations. We found that only treatment with Fe++ could block the action of CQ on CLK-1, and this in a dose-dependent way (Fig. 3A). No other metal either blocked or enhanced the effect of CQ at the metal concentrations tested (up to 30 µM). CLK-1 is expected to contain two bound iron atoms (35). Thus it is likely that the added iron competes with CLK-1 for binding to CQ, implying that the effect of CQ on CLK-1 is on its prosthetic iron. To explore this question further we also pre-treated cells with metals for 24 hours (pre-loading), followed by treatment with CQ in the absence of metals for an additional 24 hours. With this protocol only Fe++ and Co++ could be used, as Zn++ and Cu+++ were too toxic. Both metals were able to block the action of CQ (Fig. 3B). This suggests that the unique effect of iron on cells that were previously treated with CQ might be the result of greater rate of entry of iron ions into cells and mitochondria, compared to other metal ions. Given sufficient time to enter into cells cobalt appeared to be as efficient as iron in competing with CLK-1. This result suggests further that CQ acts by binding to CLK-1, possibly by chelating its iron atoms, rather than by some indirect effect, such as by affecting the intracellular pool of iron.

Treatment of RAW264.7 cells with CQ increases MCLK1 levels- We wondered whether
the treatment with CQ was destabilizing, possibly partially unfolding, CLK-1 and thus leading to an increase in its degradation. We therefore used western blotting to monitor the level of MCLK1 in RAW264.7 macrophages after treatment with CQ for 24 hours (Figure 6). We found that treatment did not reduce the levels of MCLK1, it actually increased the level of MCLK1 in a dose-dependent manner. Thus treatment with CQ does not lead to MCLK1 degradation. The increased levels of MCLK1 might be due to an increased level of synthesis of MCLK1 in response to the loss of UQ.

Treatment of C. elegans with CQ mimics a highly specific phenotype of clk-1 C. elegans mutants- We used C. elegans to further explore the effect of CQ on the function of CLK-1. In general, the most effective way to introduce compounds into worms is to add them to the agar on which worms are cultured at relatively high concentrations, expecting that a much lower concentration is reached inside the worms’ tissues. clk-1 mutants exhibit several phenotypes, including slow growth and behaviors, and an increased lifespan when fed on the standard laboratory E. coli strain OP50 (which produces UQ), but exhibit transient developmental arrest and sterility when fed on bacteria that do not produce UQ, such as the E. coli mutant strain GD1 (13). It is important to state that the latter phenotype is unique to clk-1 mutants as no other mutant tested so far shows a requirement for the presence of bacterially produced UQ (37,38). We tested the effect of CQ at 100µM on three types of worms, feeding either on GD1 or on OP50: 1) Wild type worms, which manufacture abundant endogenous UQ. 2) clk-1(e2519);rte-4(qm213) mutants in which the E to K e2519 mutation in clk-1 is suppressed by the tRNA_{glu} misense suppressor mutation rte-4(qm213) (31). These mutants, which produce only a very small amount of UQ, are expected to produce only a very small quantity of wild-type CLK-1. 3) isp-1(qm150) mutants that carry a point mutation in the ‘Rieske’ iron sulfur protein subunit of mitochondrial complex III (39). The isp-1(qm150) is essentially lethal in combination with clk-1(e2519) (extremely slow growth and sterility) (39). We expect that the latter two genotypes would be hypersensitive to inhibition of CLK-1. We found that all three types of worms showed profoundly impaired growth in the presence of CQ (Fig. 4A). The wild type worms were generally the least affected, and the clk-1;rte-4 mutants the most severely affected. Wild type worms frequently succeeded in growing into adults, but these had a severely reduced brood size (Fig. 4B), whereas the clk-1;rte-4 and the isp-1(qm150) mutants arrested development as early larvae. In contrast to what we observed on GD1, all three types of worms grew normally on OP50 and we did not detect any developmental, behavioral or aging phenotypes in response to CQ treatment. This dramatically demonstrated the specificity of the effect of CQ on worms, as it can be observed only in the absence of dietary UQ.

There are many potential reasons for the absence of an aging phenotype on UQ-replete bacteria: 1) under our conditions CQ might not inhibit the second function of CLK-1 sufficiently to mimic all aspects of mutational inactivation, although it is sufficient to inhibit growth on GD1; 2) endogenous UQ synthesis deficiency, which we do not achieve with CQ (see below), might participate in the increased lifespan of the mutants; 3) the high level of CQ we need to use in these experiments might have deleterious effect unrelated to CLK-1 inhibition.

We also tested whether the effect of CQ could be inhibited by treatment with iron, as in cells (Fig. 5). We could not test cobalt as this metal proved toxic to the worms at relevant concentrations (≥12.5µM). We tested clk-1(e2519);rte-4(qm213) mutants because they are the most sensitive to CQ and thus requiring a unequivocal blocking effect, and we used Fe^{++} at a concentration of 25µM, as CQ is believed to chelate metals in a 4:1 ratio (four molecules of CQ needed to chelate one atom of metal). We found that iron completely blocked the effect of CQ on growth (Fig. 5).

Treatment of C. elegans with CQ does not inhibit the DMQ hydroxylase activity of CLK-1- The inability of CQ to induce a phenotype on OP50 was surprising, as clk-1 mutants normally show a variety of phenotypes on OP50 (1). To explore this further we determined the levels of endogenous UQ and DMQ in wild type animals grown on OP50 in the presence of CQ at 100µM, the same concentration as used with GD1 (Fig. 4C). Worm and bacterial UQ and DMQ can be distinguished on the basis of elution time by HPLC owing to the different lengths of their isoprenoid side chains (Fig. 1A and Fig. 2). Surprisingly, we found that the levels of endogenous UQ and DMQ were not affected by
CQ (Fig. 4C), suggesting that the concentration of CQ that could be achieved in the animals with our methods was not sufficient to inhibit the DMQ hydroxylase activity of CLK-1. This, like previous evidence, suggests that CLK-1 participates also in a second, distinct, process that can be affected independently of its DMQ hydroxylase function and that is necessary for growth in the absence of dietary UQ. As UQ is found in abundance in all biological membranes, one possibility is that CLK-1 is necessary for the redistribution of UQ to other membranes after its biosynthesis in the mitochondria. Under this hypothesis, an abnormally low level of CLK-1 function would prevent endogenous UQ to reach a membrane compartment that can be reached by exogenous UQ, which is taken up from the extracellular compartment.

*Treatment of mice with CQ mimics the effect of reduced MCLK1 levels*—Having shown that CQ was capable of acting on a second function of CLK-1 in worms we sought to substantiate a similar action in mice. Heterozygous *Mclk1* mutants, which show an increased lifespan, contain normal levels of UQ (2,3,11), suggesting that the function of CLK-1 that is relevant for lifespan in mice is also not the function that is necessary for UQ synthesis. Scoring lifespan in response to drug treatment in mice is of course a long-term undertaking. However, we found that *Mclk1*+/− mouse mutants, which have normal levels of UQ (Fig. 7A), display a decrease in the activity of mitochondrial aconitase (Fig. 7C) but not of cytoplasmic aconitase (Fig. 7D), and that this effect is exactly mimicked by oral treatment with CQ for either 5 days (Fig. 7, E and F) or for only 24 hours (Fig. 7, G and H) at doses that do not induce any change in the levels of UQ (Figure 7B). The dose of CQ used (40mg/kg) was similar to those found to be sufficient for relief in animal models of neurodegenerative diseases. The decrease in mitochondrial aconitase is consistent with other findings in worms (40) and in mice (3) that suggest that there is an increase in mitochondrial but not cytoplasmic oxidative stress when the activity of CLK-1/MCLK1 is reduced.

**DISCUSSION**

*CQ acts on both functions of CLK-1*—Treatment with CQ affects the DMQ hydroxylase function of mammalian CLK-1, as indicated by the finding that treatment of mammalian cells with the compound results in a decrease of UQ levels and the appearance of DMQ. In addition, CQ affects a second function of CLK-1/MCLK1, as treatment of worms and mice with CQ at concentrations that do not affect the DMQ hydroxylase activity mimics the phenotypes associated with the loss of CLK-1/MCLK1. The following considerations suggest a mechanism for this pattern of sensitivity. First, the loss of mitochondrial aconitase activity, and the presence of other phenotypes in *Mclk1*+/− mutants (3), where MCLK1 levels are only reduced ~2-fold (11), suggests that the second function of MCLK1 involves a stoichiometric interaction with other mitochondrial proteins. Second, the nature of CQ as a metal chelator and its sensitivity to metal ions for its effect on MCLK1 suggests that its action is not that of a classical small molecular weight inhibitor that might, for example, prevent access of the substrate to a substrate-binding site. Taken together these considerations suggest that CQ might be affecting the interaction of CLK-1/MCLK1 with another protein. Thus, at low concentration, the effect of CQ on CLK-1/MCLK1 might be sufficient to decrease the level of binding to other proteins, but higher concentrations are necessary to abolish the enzymatic activity sufficiently to result in the accumulation of DMQ.

*A function of CLK-1 that is independent of UQ biosynthesis*—There are strong reasons to believe that CLK-1 has a second function in addition to that of a DMQ hydroxylase. 1) In worms, the severity of the phenotype of *clk-1* mutants does not correlate with the level of UQ (31). 2) In mice, the loss of one copy of *Mclk1* leads to numerous phenotypes in the absence of any change in UQ levels (Fig. 7A)(3). 3) Our findings with worms in the present study indicate that the reason for which *clk-1* mutants cannot grow on bacteria that do not contain UQ₈ is not because *clk-1* mutants lack endogenous synthesis of UQ₈. Indeed, the treatment with CQ that prevents growth on UQ₈-deficient bacteria does not affect the level of UQ₈ in worms (Fig. 4C). What might this second function be? The specific inability to grow on UQ₈-deficient bacteria suggests that the second function also involves UQ. One hypothesis that provides for a consistent interpretation of the various findings, and takes into account that UQ has biological functions in membrane systems other than the mitochondrial inner membrane (41), is that CLK-1 is involved in the transport of UQ from the mitochondrial inner membrane, where it
is biosynthesized, to at least one other compartment where it is needed for growth (Fig. 8). The hypothesis also suggests that this compartment can be reached directly by dietary UQ, which presumably does not need to transit through the mitochondria. Thus, for normal growth, worms need to possess either the second function of CLK-1 or receive dietary UQ. The level of CQ achieved in the worms when these are treated on plates is insufficient to inhibit the DMQ hydroxylase activity (Fig. 4C), but nonetheless prevents growth (Fig. 4A). Thus, when worms are treated with CQ in the presence of dietary UQ (OP50 bacteria) they can thrive because the crucial non-mitochondrial compartment receives sufficient UQ in the form of dietary UQ, in spite of the absence of transport from the mitochondria. When worms are treated with CQ in the absence of dietary UQ (GD1 bacteria) they cannot thrive in spite of their ability to manufacture UQ because the amount of CQ is sufficient to abolish UQ transport to the non-mitochondrial compartment.

Interaction of CQ with metal cations- The ability of iron and cobalt ions to prevent the action of CQ on the DMQ hydroxylase activity of CLK-1 suggests that chelation is important for the action of CQ on CLK-1. While it is unlikely that any Co++ is part of the structure of CLK-1, which has all the hallmarks of a di-iron carboxylate protein, which bind two iron atoms (35), Co++, like Fe++, could compete with CLK-1 for CQ binding. In this model, in the absence of an excess of metal ions, CQ would affect CLK-1 by chelating its two iron atoms and thus preventing them from participating normally in the reactions catalyzed by the enzyme.

A model for the effect of CQ on neurodegenerative diseases- Our findings suggest a model for how CQ might act on neurodegenerative diseases (Fig. 8). The known effect of CQ on cancer cells is not included in the model as its characteristics, which demonstrably involves transport of Zn++ and Cu++ into cells, are too distinct from those of the effect on CLK-1 (21,22). Our model suggests that at low concentration CQ affects a function of CLK-1 that is distinct from UQ biosynthesis, which is responsible for its effect on the rate of aging, at least in mice (2,3). Thus, although the effect of CLK-1 on neuronal function has not yet been tested, we hypothesize that the widespread effect of CQ on mouse models of age-dependent neurodegenerative diseases could be the result of its effect on the rate of aging via CLK-1. Although it is not yet well understood how aging leads to age-dependent diseases (8), the availability of a compound (CQ) and a target (CLK-1) that link the two will be valuable in the current effort to apply aging research to medicine.

REFERENCES


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**FIGURE LEGENDS**

**Fig. 1** Clioquinol (CQ) reversibly inhibits the demethoxyquinone (DMQ) hydroxylase CLK-1 in mouse and human cells. A) Mouse macrophage-like RAW264.7 cells or human Hela cells were treated with CQ and quinones were extracted and separated on a reverse-phase HPLC column. Treatment with CQ for 24 hours reduces the size of the peak corresponding to ubiquinone (UQ) and produces the appearance of a large peak corresponding to DMQ. A very small peak corresponding to DMQ is also generally seen in untreated cells. In mouse cells the main UQ is UQ9 (where the 9 indicates the length of the isoprenoid side chain). In human cells UQ10 is the principle UQ found. We assume that the peak that elutes just before UQ10 in Hela cells treated with CQ is DMQ10. B) The inhibition of CLK-1 by CQ in RAW264.7 cells is dose-dependent in the range of 1-15 μM. At higher doses, CQ showed cytotoxicity (data not shown), which caused a decline of both DMQ9 and UQ9 levels. Data from 3 independent experiments are shown and represented as means and standard deviations. C) The effect of CQ on CLK-1 is quickly reversible upon removal of the compound from the medium. 2 hours after removal of the drug DMQ9 levels return to those found in untreated RAW264.7 cells. Data from 3 independent experiments are shown and represented as means and standard deviations.

**Fig. 2** CQ inhibits CLK-1 but not UbiF. HPLC chromatographs of quinones extracted from bacterial strains with and without treatment with CQ are shown. A) JF496 (DE3) deficient in UbiF function. B) JF496(DE3) transformed with an empty vector pET16b (Novagen). C) JF496 (DE3) transformed with a plasmid carrying and expressing *ubiF*. D) JF496(DE3) transformed with a plasmid expressing *Mclk1*. E) JF496(DE3) transformed with the plasmid carrying *ubiF* after exposure to 20μM CQ for 6 hours. F) F496(DE3) transformed with pET16b-Mclk1 after exposure to 17.5μM CQ for 6 hours. Treatment with CQ does not affect UQ biosynthesis in JF496 bacteria that express *ubiF*. It only affects UQ biosynthesis in bacteria expressing *Mclk1*.
**Fig. 3** A) Iron ions block the inhibition of CLK-1 by CQ. Various metal ions (Fe++, Cu++, Zn++ and Co++) were added into the culture medium of RAW264.7 cells previously treated with CQ for 24 hours. Only Fe++ could block the inhibitory effect of CQ on CLK-1, and in a dose-dependent fashion. Other metal ions, added at up to 2 times the CQ concentration had no effect on the action of CQ on CLK-1. B) Pre-loading of RAW264.7 cells with Co++ prevented the inhibition of CLK-1 by CQ to a similar degree as pre-treatment with Fe++. The effect of Zn++ and Cu++ on the action of CQ could not be assayed due to their strong cytotoxicity in this protocol. These results show that both cobalt and iron, if provided to the cells for sufficient time to increase cellular concentration, are able to block CQ’s action on CLK-1.

**Fig. 4** CQ treatment of *C. elegans* mimics specific aspects of the *clk-1* phenotype without inhibiting UQ biosynthesis. A) Wild type worms or worms carrying *isp-1(qm150)*, a mutation that is synthetically lethal in combination with *clk-1* mutations, or worms carrying *clk-1(e2519); rte-4(qm213)*, which are *clk-1* mutants suppressed by a tRNA missense suppressor and that are believed to contain only very small levels of wild-type CLK-1 protein, all are able to grow on UQ-deficient (GD1) bacteria, but show profoundly impaired growth in the presence of CQ. Treated worms appear smaller than untreated worms because they are still at younger, larval, developmental stages. In contrast, they all grow normally in the presence of CQ when feeding on bacteria (OP50) containing UQ (only pictures for the wild type are shown for this situation). The pictures show the time points at which the largest difference between treatments could be observed. The experiment was repeated several times and the growth rate of the wild type is generally the least, and that of *clk-1(e2519); rte-4(qm213)* mutants the most severely affected. B) Wild type worms that succeed in reaching adulthood and become fertile when feeding on UQ-deficient bacteria (GD1) in the presence of 100 μM CQ show a reduced brood size. Brood size was scored by counting all the progeny of individual worms. N=11 for worms feeding on OP50, and N=11 and N=13 when feeding on GD1 in the presence of DMOS only or CQ, respectively. C) Wild type worms grown on OP50 (UQ-containing bacteria) in the presence of CQ produce normal levels of UQ9 and DMQ9, indicating that the UQ-biosynthesis function of CLK-1 is not inhibited at the concentration of CQ that is achieved in the worms’ cells by the treatment. Thus, the effect of CQ on growth on UQ-deficient bacteria involves an activity of CLK-1 that is distinct from its function in UQ biosynthesis.

**Fig. 5** Iron blocks the effect on CQ on worms. *clk-1(e2519);rte-4(qm213)* mutants at the L1 stage were placed on CQ plates and control DMOSO plates, seeded with GD1 and supplemented or not with FeSO4 at 25μM. The growth of the worms was monitored and the pictures show the time points at which the largest difference between treatments could be observed. Affected worms appear smaller than unaffected worms because they are still at younger, larval, developmental stages. Iron completely blocked the effect of CQ on growth. The effect of cobalt could not be tested because it appeared to be toxic to worms at the relevant concentrations (≥12.5μM). *clk-1(e2519);rte-4(qm213)* mutants were used in this test as they appear to be most sensitive to CQ treatment.

**Fig. 6** Treatment with CQ increases MCLK1 levels. Whole cell lysates were subjected to Western blot analysis using antibodies against MCLK1, and porin, which was used as a loading control. The Western blot analysis shows that treatment with CQ did not lead to MCLK1 degradation, in fact, it elevated MCLK1 levels in a dose-dependent manner.

**Fig. 7** Treatment of mice (C57BL/6) with CQ mimics the phenotype observed in *Melk1*+/− mutant mice: a reduction of mitochondrial, but not of cytosolic, aconitase in the liver in the absence of any effect on UQ levels. *Melk1*+/− in the C57BL/6 background (2) show reduced mitochondrial (A) but not cytosolic aconitase (B). Clioquinol was administered by gavage for 1 or 5 days, at 40mg/kg/day. Both treatments have a similar effect of reducing mitochondrial aconitase activity (C, E) and not affecting cytosolic aconitase activity (D, F). However, neither *Melk1* heterozygosity (G), nor CQ (H) treatment affects the level of UQ9.

**Fig. 8** A model of the action of CQ on CLK-1/MCLK1 and its physiological consequences. Our results imply that a high effective concentration of CQ can inhibit the DMQ hydroxylase activity of CLK-1.
likely via its properties as a chelator. Co^{++} and Fe^{++} can effectively block the action of CQ on CLK-1 by competing with CLK-1 for binding to CQ. At lower effective concentrations CQ is still capable of inhibiting a second function of CLK-1 that we hypothesize to be involved in the transport of UQ from the mitochondria to at least one extra-mitochondrial compartment where sufficient UQ is required for the growth of the animals. This compartment can be provided with UQ either via transport from the mitochondria or via a dietary source. Furthermore, the function of this compartment impinges on the rate of aging, as genetic reduction of CLK-1 activity in worms and in mice prolongs lifespan. Thus, we hypothesize that the effect of CQ on several distinct neurodegenerative diseases is via the age-dependency of these pathologies.
Figure 1
Wang et al.

A

Mouse RAW264.7 macrophages 24 hours treatment

Human HEla cells 24 hours treatment

B

UQ ng/mg of protein vs CQ (µM)
Figure 1
Wang et al. Continued

![Bar chart showing the levels of UQ and DMOQ as a function of time after removal of CQ. The x-axis represents time (0 min, 15 min, 30 min, 60 min, 120 min), and the y-axis represents UQ ng/mg of protein. The chart indicates a increase in UQ levels over time, particularly noticeable at 120 min.]
Figure 2
Wang et al.

A

DMQ₈

uᵦᵣᵦF mutant

UQ₈

B

DMQ₈

uᵦᵦᵦF mutant

UQ₈

C

uᵦᵦᵦF mutant

+ uᵦᵦᵦF wt gene

D

uᵦᵦᵦF mutant

+ Mclᵦ₁ wt gene

E

uᵦᵦᵦF mutant

+ uᵦᵦᵦF wt gene

+ CQ treatment

F

uᵦᵦᵦF mutant

+ Mclᵦ₁ wt gene

+ CQ treatment
Figure 3
Wang et al.

A

[Graph showing UQ oligomer levels in cells treated with various concentrations of metal ions and CQ.]

B

[Graph showing UQ oligomer levels in cells treated with different metal ions and DMSO.]

- DMQ₉
- UQ₉

Figure legend:
- UQ oligomer levels are measured in ng/mg of protein.
- Metal ions include FeSO₄ and CuSO₄.
- DMSO is used as a control.

Experiment details:
- CQ 24h after addition (no metal added)
- 2h after CQ removal
- Various concentrations of FeSO₄ and CuSO₄.
**Figure 4**

**A**

<table>
<thead>
<tr>
<th>Condition</th>
<th>0.5% DMSO</th>
<th>100µM Clioquinol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
</tr>
<tr>
<td>isp-1(qm150)</td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
</tr>
<tr>
<td>clk-1(e2519); rte-4(qm213)</td>
<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
</tr>
<tr>
<td>Wild type</td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
</tr>
</tbody>
</table>

**B**

![Graph showing brood size](image9)

- **0.5% DMSO**
- **100µM Clioquinol**

**OP50**

- **UQ-replete**
- **E. coli**

**GD1**

- **UQ-deficient**
- **E. coli**
Figure 4
Wang et al.
Continued
Figure 5
Wang et al.

0.5% DMSO

100µM Clioquinol

No added Fe^{++}

25µM Fe^{++}
Figure 6
Wang et al.
Mitochondrial aconitase activity

A

UQ₉ in Mclk1 +/- mice

Relative to mean Mclk1 +/+ levels (%)

Mclk1 +/+ Mclk1 +/-

B

UQ₉ after clioquinol treatment

Relative to mean vehicle levels (%)

Vehicle Clioquinol

C

Mitochondrial aconitase activity

Relative to mean Mclk1 +/+ activity (%)

Mclk1 +/+ Mclk1 +/-

p<0.05

D

Cytosolic aconitase activity

Relative to mean Mclk1 +/+ activity (%)

Mclk1 +/+ Mclk1 +/-

E

5 day treatment

Mitochondrial aconitase activity

Relative to mean vehicle activity (%)

Vehicle Clioquinol

p<0.02

F

5 day treatment

Cytosolic aconitase activity

Relative to mean vehicle activity (%)

Vehicle Clioquinol

G

24 hours treatment

Mitochondrial aconitase activity

Relative to mean vehicle activity (%)

Vehicle Clioquinol

p<0.01

H

24 hours treatment

Cytosolic aconitase activity

Relative to mean vehicle activity (%)

Vehicle Clioquinol
Figure 8
Wang et al.

DMQ
UQ
Fe²⁺ or Co²⁺

CLK-1
DMQ hydroxylase
UQ transport?

Mitochondria

Exogenous UQ → UQ → Extra-mitochondrial compartment

GROWTH
AGING

? → NEURODEGENERATION
The anti-neurodegeneration drug clioquinol inhibits the aging-associated protein CLK-1

Ying Wang, Robyn Branicky, Zaruhi Stepanyan, Melissa Carroll, Marie-Pierre Guimond, Abdelmadjid Hihi, Steve Hayes, Kevin McBride and Siegfried Hekimi

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