# Cardiac light chain amyloidosis: The role of metal ions in oxidative stress and mitochondrial damage

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Cardiac light chain amyloidosis: The role of metal ions in oxidative stress and mitochondrial damage

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Abbreviated Title: Metal ions drive cardiac light chain toxicity

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ABSTRACT

Aims: The knowledge of the mechanism underlying the cardiac damage in immunoglobulin light chain (LC) amyloidosis (AL) is essential to develop novel therapies and improve patients’ outcome. Although an active role of radical oxygen species (ROS) in LC-induced cardiotoxicity has already been envisaged, the actual mechanisms behind their generation remain elusive. This study was aimed at further dissecting the action of ROS generated by cardiotoxic LC in vivo and investigating whether transition metal ions are involved in this process. In absence of reliable vertebrate model of AL we employed the nematode Caenorhabditis elegans, whose pharynx is an “ancestral heart”. Results: LC purified from patients with severe cardiac involvement intrinsically generated high levels of ROS and, when administered to C. elegans induced ROS production, activation of the DAF-16/FOXO pathway and expression of proteins involved in stress resistance and survival. Profound functional and structural ROS-mediated mitochondrial damage, similar to that observed in amyloid-affected hearts from AL patients, was observed. All these effects were entirely dependent on the presence of metal ions since addition of metal chelator or metal-binding 8-hydroxyquinoline compounds (Chelex, PBT2 and clioquinol) permanently blocked the ROS production and prevented the cardiotoxic effects of amyloid LC. Innovation and Conclusion: Our findings identify the key role of metal ions in driving the ROS-mediated toxic effects of LC. This is a novel conceptual advance which paves the way for new pharmacological strategies aimed at not only counteracting but totally inhibiting the vicious cycle of redox damage.
INTRODUCTION

Organ damage in immunoglobulin light chain (LC) amyloidosis (AL) results from the direct toxic effects of aberrant, misfolded monoclonal LC and from the structural subversion caused by extracellular amyloid deposits in target organs (33,34). Approximately 75% of patients manifest heart involvement at presentation. These patients experience rapid worsening of cardiac failure with a median survival of only 6 months if cytotoxic chemotherapy fails to stop plasma-cell LC production. Patients with advanced cardiac involvement (53) are frequently too fragile to tolerate chemotherapy: paradoxically, patients most in need of treatment are those that at present cannot be treated effectively.

In patients in whom chemotherapy reduces the concentration of the amyloidogenic circulating LC, cardiac dysfunction improves—despite the amyloid load remaining unaltered—suggesting that both host-related factors and intrinsic LC characteristics are required to cause organ toxicity. No clear relationship has been established between the LC germline genes and the targeting of specific organs, including the heart, and no specific features related to LC cardiotoxicity have been described yet, rendering the protein’s ability to target the heart impossible to predict (39). A better understanding of the molecular and biochemical mechanisms underlying the cardiotoxicity of LC is essential to design innovative therapeutic strategies and improve patients’ outcome.

The processes through which extracellular LC leads to cardiac pathology are still under investigation. Data obtained in vitro suggests that LC associated with cardiomyopathy has an intrinsic and specific cardiotoxic potential and involves different processes such as increased apoptosis, oxidative stress and the activation of specific signal transduction pathways (8). In order to develop novel therapeutic strategies, facilitate early diagnosis and investigate the
mechanisms of toxicity, the development of experimental models that are able to recapitulate LC cardiotoxicity remains a major and urgent need. Great efforts have been spent over the years to generate appropriate animal models that recapitulate the heart-specific toxicity of LC. Despite multiple attempts by different groups, no transgenic vertebrate animals expressing human LC have been obtained. Experiments performed on isolated mouse hearts have shown that the infusion of LC purified from patients with cardiac involvement increases end-diastolic pressure (30). Similar results were obtained in zebrafish in which the injection of LC from patients with cardiac involvement caused a reduction in cardiac output and early mortality (35). New hints come from studies recently performed on Caenorhabditis elegans, a valuable animal model for investigating the pathogenic effects of monoclonal LC in vivo (13). The pharynx of this nematode is an “ancestral heart” (31), evolutionarily related to the vertebrate heart. Its muscle cells have autonomous contractile activity, reminiscent of cardiac myocytes, and the electrical coupling between muscle cells, calcium-based action potentials and high mitochondrial density resemble those present in mammalian heart (3,47,48,56). The rhythmic contraction and relaxation of the nematode’s pharyngeal muscle is responsible for the ingestion and transport of food from the mouth to the intestine. Stress-induced inhibition of feeding was suggested as an important survival mechanism that limits the intake of toxic solutes. In fact, pharyngeal pumping is inhibited by chemical stressors that induce the production of cellular stress proteins. This nematode-based approach offers many advantages; it is more rapid, less expensive and avoids the ethical issues involved with the use of vertebrate animals such as rodents and zebrafish. In addition, the small amount of human LC required to perform the analysis renders this assay feasible for future routine clinical analysis. All these features render C. elegans as the most feasible approach for studying the mechanisms of LC-induced toxicity in
vivo and screening new drugs. The propensity of cardiotoxic LC to generate reactive oxygen species (ROS) rendered these proteins recognizable as “stressors” by worms (13). Cardiotoxic LC also enhanced ROS production by mitochondria, causing permanent pharyngeal dysfunction and reducing lifespan. Importantly, administration of anti-oxidant drugs N-acetyl-cysteine (NAC) and tetracycline hydrochloride (TETRA), prevented these effects (13) indicating that ROS generated by amyloidogenic cardiotoxic LC actively contribute to their detrimental activity in vivo. Strategies aimed at halting the vicious cycle of oxidative stress might provide innovative and potentially less toxic options, specifically aimed at at not only counteracting but totally inhibiting the vicious cycle of redox damage.

We here report that ROS produced by cardiotoxic LC act as signaling molecules in C. elegans, activating the Forkhead transcription factors (FOXO)/DAF-16 pathway (37) and controlling genes involved in stress resistance and survival. LC also caused a remarkable ultrastructural alteration in the pharynx of worms and mitochondrial damage similar to that observed in amyloid-affected hearts from AL patients.

Metal ions play a key role in driving all these effects suggesting metal-binding agents as a compelling pharmacological strategy for AL. The metal cheletaing resin Chelex and two transition metal-binding compounds, members of the 8-hydroxyquinoline chemical class, were considered: clioquinol (5-chloro-7-ido-quinolin-8-ol, CQ) and PBT2 (44). CQ is a legacy compound while PBT2 is a novel non-iodinated analog which has been shown to assist in restoration of metal homeostasis (44). All compounds reduced the ability of cardiotoxic LC to generate ROS and counteracted their related functional and structural damage on the worm’s pharynx. Notably, the administration of pharmacologic low doses of PBT2 in combination with
tetracyclines, which reduced early deaths in patients with cardiac AL when added to standard chemotherapy (52), resulted in a synergistic beneficial effect, highlighting the potential application of this pharmacological strategy for cardiac AL patients.
RESULTS

Metal ions drive ROS generation and pharyngeal damage

The pharyngeal *C. elegans* impairment caused by the administration of cardiotoxic LC, at 100 µg/mL, a concentration representative of serum free-LC in AL patients, was related to their capacity to generate ROS, particularly within mitochondria (13). The role of transition metal ions in regulating the ability of LC to produce ROS was investigated here. To this end, monoclonal LC were purified from 3 patients with cardiac AL and from 3 negative control patients with multiple myeloma (Supplementary Table S1).

Cardiotoxic LC, already reported to produce a greater amount of oxygen radicals, particularly •OH radical species, compared to myeloma (13), intrinsically generated significantly higher levels of H$_2$O$_2$ (725 ± 55.8 vs. 278 ± 61.9 fluorescence intensity (FI) value for cardiotoxic LC and myeloma, respectively, p<0.01, Fig. 1A). ROS generation was significantly attenuated when metal ions were chelated eluting protein on Chelex, a metal-chelating resin (Fig. 1A). In particular, Chelex reduced the H$_2$O$_2$ generation by cardiotoxic LC of 69% (725 ± 55.8 Fluorescence Intensity (FI) in cardiotoxic LC and 222 ± 101 FI in cardiotoxic LC+ Chelex) and fully abolished its production by myeloma (278 ± 61.9 FI in myeloma and 4.17 ± 4.09 in myeloma + Chelex) (Fig. 1A). The residual ROS produced by cardiotoxic LC after Chelex exposure might be due to partial removal of metals, since Chelex is notoriously unreliable at removing trace metals. Metal chelation did not affect the secondary structure content and thermostability of LC (Supplementary Fig. S1).

The specific involvement of H$_2$O$_2$ on the *C. elegans* impairment caused by the administration of cardiotoxic LC was investigated using catalase. To this end, cardiotoxic and myeloma proteins were incubated with 100 U/mL of catalase for 15 min before
administration to worms. Catalase, at this concentration, was effective to completely
abolished the pharyngeal dysfunction induced by 1 mM H₂O₂ and counteracted the
toxicity caused by cardiotoxic LC but not myeloma feeding (Supplementary Fig. S2).
When cardiotoxic proteins purified from patients with severe amyloid cardiomyopathy
were treated with Chelex and administered to worms in metal-free water, their ability to
cause pharyngeal dysfunction was abolished whereas no change was observed when
myeloma proteins were eluted on Chelex (Fig. 1B). Similar results were obtained when
LC were co-administered in metal-free water with the generic metal chelator EDTA (17)
(Fig. 1B).

The addition of copper and iron, but not zinc, to cardiotoxic and myeloma proteins treated
with Chelex restored their native ability to produce H₂O₂ (Fig. 1C). When copper was
added to the protein solution, the pharyngeal dysfunction induced by cardiotoxic LC
worsened, whereas iron and zinc did not exert any additional effect (Fig. 1D). A similar effect was
observed when copper was administered together with Chelex-treated cardiotoxic protein
(Supplementary Fig. S3). The pumping rate of C. elegans fed myeloma protein was not affected
by either copper or iron and zinc (Fig. 1D). These results indicate that both iron and copper
can drive the generation of H₂O₂ by cardiotoxic and myeloma proteins in cell free
conditions, but only copper exerts an active role in the pharyngeal toxicity.
Copper has already been reported to be able to bind to amyloidogenic LC (12). To search
for a direct interaction of copper with LC, near and far-UV CD spectra were recorded, as well as
tryptophan fluorescence measurements. No significant modifications indicative of direct binding
of copper to cardiotoxic and myeloma proteins were observed (data not shown), suggesting that
the interaction of this metal with LC is transient, local and relatively weak.
However, the increase in $\text{H}_2\text{O}_2$ production by cardiotoxic LC in the presence of copper proves that the metal was reduced from $\text{Cu}^{2+}$ to $\text{Cu}^+$ by the protein, suggesting that proteins must possess reducing species (most commonly thiols such as cysteine) to donate electrons (38). The pre-incubation of cardiotoxic LC with iodoacetamide—which links covalently with the thiols and thus prevents them from being a source of electrons (54)—cleared the copper-induced increased production of $\text{H}_2\text{O}_2$ (Fig. 1E), demonstrating that an interaction between protein and copper is necessary for radical production. Electron paramagnetic resonance (EPR) measurements performed on cardiotoxic LC show that copper enhances the radical production, although a linear dependence between copper concentration and amount of radical could not be established. This is likely due to the fact that the redox cycling process is catalytic; hence, it does not follow linear kinetics (Supplementary Fig. S4).

Furthermore, we observed that the feeding of *C. elegans* with cardiotoxic LC, but not myeloma, resulted in a specific increase in endogenous copper levels (Supplementary Fig. S5A), whereas iron and zinc ions levels were not significantly altered (Supplementary Fig. S5B).

*Metal-binding compounds counteracted the cardiotoxic LC-induced functional and structural damage on the worm’s pharynx*

The effect of CQ and PBT2 was considered. Their effect in counteracting the pharyngeal dysfunction caused by cardiotoxic LC was dose-dependent (Fig. 2A), PBT2 being significantly more effective than CQ, with an $\text{IC}_{50}$ approximately seven thousand-fold lower ($\text{IC}_{50}$: $1.08 \pm 1.1$ nM and $7.5 \pm 1.0$ µM for PBT2 and CQ, respectively; $p<0.01$ Student’s t test). We settled on an optimal concentration of 25 µM for CQ and 2 nM for PBT2: dose levels that caused a 98% reduction of $\text{H}_2\text{O}_2$ production (Fig. 2B) and completely abolished the
pharyngeal impairment caused by all cardiotoxic LC under investigation (Fig. 2C). At these concentrations CQ and PBT2 did not affect the secondary structure content and thermostability of LC (Supplementary Fig. S1). Noteworthy, CQ and PBT2 exerted similar effects on both natural BJ and recombinant LC from the same cardiac amyloid patient (Supplementary Table S2) indicating that the particular ability of amyloid cardiac LC to interact with metal ions was not an artefact of the protein purification procedure. CQ and, to a lesser extent PBT2, counteracted the cardiotoxic LC-induced elevation of copper levels in worms, without affecting the levels of iron or zinc (Supplementary Fig. S5). CQ binds iron with moderate affinity, but PBT2 is a poor ligand for iron (43). Because both compounds bind copper with similar affinity, this element is the most likely redox-active binding partner involved in cardiotoxic LC-induced dysfunction.

Neither compound counteracted H$_2$O$_2$-induced pharyngeal toxicity (Fig. 2C), indicating that their protective effect against cardiotoxic LC is not related to a general anti-oxidant activity. This conclusion was further supported by the fact that CQ and PBT2 protected nematodes against the increase in mitochondrial ROS generation caused by cardiotoxic LC but not H$_2$O$_2$ (Fig. 2D). Drugs alone did not modify the pumping rate (Fig. 2C), nor the increase of pharyngeal mitochondrial oxygen burden (Fig. 2D).

As already reported (13), the exposure of *C. elegans* to cardiotoxic LC significantly reduced their life-span (median survival: 13 days and 9 days for vehicle- and cardiotoxic LC-fed worms, respectively, p=0.0001, Log-rank test) (Fig. 2E). A single dose of 25 µM CQ and administration of 2 nM/day PBT2 significantly prolonged the survival of cardiotoxic LC-treated worms, restoring their natural lifespan (median survival: 14 days for cardiotoxic LC+ CQ treated worms (p=0.036 vs. cardiotoxic LC) and 13 days for cardiotoxic LC+ 2 nM
We investigated whether ROS produced by cardiotoxic LC caused alterations in pharyngeal sub-cellular compartments, particularly mitochondria, which play a vital role in providing energy for contractile activity. Transmission electron microscopy (TEM) analyses showed that the pharyngeal muscles of worms fed cardiotoxic LC, but not myeloma protein, resulted in profound alteration of the pharyngeal ultrastructure and caused mitochondrial damage compared with vehicle treated nematodes (Fig. 3A-C). These morphological alterations were accompanied by impaired mitochondrial function, as demonstrated by the decreased membrane potential, which was determined using the fluorescent probe tetramethylrhodamine, methyl ester (TMRM) (Supplementary Fig. S6).

Since similar features were observed when worms were fed all the cardiac amyloid LC considered in the study, which derived from unrelated germline gene, this indicated that the observed functional and structural effects were strictly dependent on features that are intrinsic to cardiac LC, with no restriction to a particular germline gene or set of genes.

The subcellular alterations observed in worms were comparable to damage caused by cardiac LC in human heart tissue as proved by endomyocardial biopsies from AL patients with advanced cardiac dysfunction (Supplementary Table S3) analyzed by TEM. Similar to worms exposed to cardiac LC, most human mitochondria showed dramatic structural derangement (Fig. 4A-C and Supplementary Fig. S7): their size was enlarged and also the cristae formed by the internal membrane were almost totally lost. In contrast, endomyocardial biopsies from subjects who had undergone heart transplantation for primary dilated cardiomyopathy (used as controls for disease and severity of heart dysfunction), showed fully preserved mitochondria, both in size and morphology, and only scattered
mitochondria with minor alterations (Fig. 4D). These results, resembling those reported by Guan et al. (21), lend support to the validity of the nematode model and reinforce the rationale of its use for designing and testing new therapeutic approaches.

When administered to \textit{C. elegans}, PBT2 and CQ were capable of protecting the pharyngeal cells from mitochondrial-induced damage (Fig. 3D-G). A similar effect was observed with 50 µM TETRA, an antibiotic which has also antioxidant and metal ion chelator activity (10,49), and 5 mM NAC, a prototypic antioxidant compound, which were both capable of neutralizing the ROS generation (13) and pumping dysfunction caused by cardiotoxic LC (Supplementary Fig. S8). These findings indicated that ROS are responsible for the specific ability of cardiotoxic LC to damage sub-cellular pharyngeal structures, particularly mitochondria.

\textit{Metal ions drive the ability of cardiotoxic LC to regulate genes involved in oxidative stress resistance}

In \textit{C. elegans} an increase in ROS levels can result in the activation of the insulin/insulin growth factor-1 (I/IGF-1) signaling pathway (4,23), driving the translocation of the FOXO/DAF-16, which actively controls diverse target genes involved in oxidative stress resistance and survival (37). We observed that ROS generated by cardiotoxic LC act as signaling molecules modulating the FOXO signaling pathway. Under basal conditions DAF-16 was mainly localized in the cytosol of transgenic TJ356 \textit{C. elegans} nematodes expressing GFP under control of \textit{daf-16} promoter (23) (Fig. 5A). The administration of cardiotoxic LC caused a significant increase of the nuclear translocation of DAF-16, detectable as the appearance of condensed green foci in the bodies of the worms (Fig. 5B).
1. The nuclear localization was confirmed by the colocalization of the blue fluorescent dye
specific for nuclei (Supplementary Fig. S9). A similar effect was observed when
nematodes were fed H$_2$O$_2$ as a positive control (Supplementary Fig. S10A). CQ and
PBT2, counteracted the activation of DAF-16 induced by the cardiotoxic LC (Fig. 5C, D).
A similar effect was observed with 50 µM TETRA, but not with 5 mM NAC (Fig. 5C, D).

7. The adaptive responses of the antioxidant defense system were then evaluated by
evaluating the expression of two genes transcriptionally targeted by DAF-16. In particular, activation of the expression of small HSP-16.2, which can act as a ROS-sensor and also affects the lifespan of worms (23), and the activity of the antioxidant enzyme manganese superoxide dismutase SOD-3 were determined using transgenic CL2070 (23) and CF1553(2) worms, expressing GFP control of hsp-16.2 or sod-3 promoter, respectively. It is in fact known that among the five SODs encoded and expressed by C. elegans, SOD-2 and SOD-3 are MnSODs localized in the mitochondrial matrix (4,25,36). SOD-2 contributes to the SOD activity during normal development, whereas sod-3 is specifically upregulated by daf-16 gene in response to ROS-induced stress. Cardiotoxic LC, but not myeloma, caused a significant increase in HSP-16.2 (Fig. 6A, B) as well as SOD-3 expression (Fig. 6C, D) in the pharynx of nematodes, similarly to that observed with H$_2$O$_2$ (Supplementary Fig. S10B). In contrast, exposure to CQ and PBT2 significantly reduced the LC-induced HSP-16.2 and SOD-3 protein expression, as indicated by the absence of GFP signal in the pharynx of CL2070 and CF1553 worms, respectively (Fig. 6).

Overall these results indicated that cardiotoxic LC, by means of metal ion-mediated ROS production and consequent FOXO/DAF-16 pathway activation, stimulate genes involved
in the control of the oxidative stress response and lifespan, and that LC co-incubation with metal-binding compounds, such as CQ and PBT2, abolished the worm stress response.

PBT2 and tetracycline exert a synergic effect

Based on the pre-clinical observations obtained in *C. elegans* on the protective effect of tetracyclines (13), a clinical study has been recently designed aimed at evaluating the cardio-protective effect of doxycycline on the heart of AL patients. Preliminary data indicated that when doxycycline was orally administered at 100 mg twice daily (corresponding to about 100 µM/day) in addition to standard chemotherapy, the early deaths in patients with cardiac AL amyloidosis was reduced (52).

We here analyzed whether the administration of a metal-binding agent can improve the effect of tetracyclines in *C. elegans*. In anticipation of clinical application, we set out the concentration of TETRA at 20 µM, a dose lower than that used in clinic. To reflect circumstances most likely encountered in the clinic we designed experiments in which drugs were administered to worms for 30 min when the pharynx was already damaged by cardiotoxic LC. As expected, in these experimental conditions 20 µM TETRA was ineffective whereas 2 nM PBT2, but not 25 µM CQ, was capable of restoring the pharyngeal dysfunction caused by cardiotoxic LC (Fig. 7A). To determine the lowest effective dose of PBT2 able to improve the effect of TETRA, this metal-binding compound was administered at 2 nM or 0.5 nM concentrations. Interestingly, PBT2, at the ineffective concentration of 0.5 nM, when combined with TETRA, exerted a synergistic and beneficial effect (Fig. 7B). Although this treatment did not totally reverse the pharyngeal dysfunction to normal levels, these results suggest that the combined administration of low doses of this metal-binding compound and
TETRA may represent an innovative pharmacological approach to break the vicious cycle of oxidative stress and heart damage induced by cardiotoxic LC.
DISCUSSION

The 25% of patients with AL amyloidosis diagnosed with end-stage cardiac damage represent an unmet need, since no available therapy can improve their prognosis (34,53).

Innovative approaches are needed in order to counteract the rapidly progressing cardiac failure sustained by the direct cardiotoxicity of amyloid LC.

We performed this study to gain insights into the mechanisms by which LC can damage the hearts of AL amyloidosis patients exploiting the robust C. elegans model. The results obtained indicate that ROS both result from and promote pharyngeal oxidative damage caused by cardiotoxic LC (Fig. 8). This effect was not related to the germline gene, which is different for each LC considered, nor to the method applied to obtain the proteins, but was strictly related to the specific ability of cardiotoxic LC to interact with metal ions and produce ROS.

Metal ions, particularly copper, have previously been shown to modulate LC polymerization (12) and interact with different amyloidogenic proteins, such as β-amyloid, prions and α-synuclein (24,41), promoting misfolding and generating toxic oligomeric assemblies (32). We have demonstrated here that the presence of redox-active transition metals, particularly copper, is required to drive ROS production by cardiotoxic LC resulting in injurious oxidative stress to C. elegans pharyngeal cells. The molecular and biochemical reasons underlying the peculiar role of copper in driving the cardiotoxic LC detrimental effects remain to be elucidated. Different activities of iron and copper in catalysing thiol oxidation (38) and the high pharyngeal concentration of iron required to cause peroxide sensitivity and oxidative damage, which is in the millimolar range (compared to the micromolar range of copper) (50), as well as the expression of specific copper protein...
chaperones in the pharynx (51), may be the source of the different effects exerted by the
two metals. The active role of copper as the most likely redox-active binding partner is also
reinforced by the fact that CQ and PBT2 bind copper with similar affinity but bind iron with
moderate or low affinity, respectively (43).

ROS generated by cardiotoxic LC, in addition to cause a behavioral response in C. elegans
which inhibited feeding (6), accumulate in the pharynx (13). Metal-mediated ROS generation
also occurs within the pharyngeal cells damaging organelle functions and the ultrastructure,
particularly at the mitochondrial level, resulting in disruption of their integrity. These cellular
responses can trigger the permanent cellular impairment and death observed in the pharynx of
worms fed cardiotoxic LC (13).

Mitochondria are naturally a rich source of ROS, usually scavenged by SOD and catalases.
In the pharynx of worms fed cardiotoxic LC in response to the oxidative stress we observed an
activation of SOD-3, a protein mainly expressed in the mitochondria of pharyngeal cells
(20,25). Much of the ROS produced by mitochondria is generated by copper and iron in the
active sites of the enzymes of the oxidative phosphorylation pathway. When
mitochondrial membrane integrity is disrupted, the oxidative phosphorylation pathway
becomes inefficient leading to extensive ROS generation which could overwhelm the cell
under conditions when it is already stressed by the toxic LC. Data obtained by our group
from human cardiac cells show that cardiotoxic LC co-localize with mitochondria and
spatially associate with selected interactors that are involved in modulating mitochondrial
morphology and function and in ATP production by fatty acid β-oxidation pathway (29).
Mitochondria of human cardiac cells exposed to cardiotoxic LC display ultrastructural
changes, supporting the concept of mitochondrial involvement (29). Furthermore,
mitochondrial damage was detected in the explanted heart from AL patients with severe cardiac involvement undergoing heart transplantation, supporting the clinical relevance of the experimental observations obtained in the C. elegans model. The observed disruptive effect of LC may be crucial for mitochondria-rich tissues such as rhythmic contractile organs, like the pharynx of C. elegans and the vertebrate heart, given the vital role of these organelles in supplying contraction energy (31).

The hypothesis that ROS has a causal role in damaging the pharynx of worms was supported by the observation that antioxidant drugs, known to counteract ROS-induced pharyngeal dysfunction, prevent the mitochondrial damage caused by cardiotoxic LC (13). ROS generated by LC can also trigger important signalling events aimed at limiting and repairing the stress-induced damage. A first line of defence is represented by the activation of chaperones, such as heat shock proteins, whose expression can be induced by ROS, metal ions and misfolded proteins (16). To this end, to gain a better insight in the molecular mechanisms triggered by the oxidative stress caused by cardiotoxic LC, we observed that they activate the expression of HSP-16.2, an αB-crystallin-related protein involved in the metabolism and toxicity of other amyloidogenic proteins (16). Importantly, we also observed the nuclear translocation of the FOXO/DAF-16 transcription factor, similarly to what happens in response to paraquat administration (9). This transcription factor, member of the evolutionary conserved I/IGF-1-like signaling pathway, regulates a set of stress-responsive genes, including hsp-16.2 and sod-3, and also controls the longevity of C. elegans. These studies indicate that metal-mediated ROS generation target mitochondria and activate several intersecting signalling pathway and genes, advancing the understanding of the mechanism underlying LC toxicity.
The effectiveness of employing a pharmacological strategy aimed at restoring the metal homeostasis and interrupt the vicious cycle of oxidative stress production caused by metal ions was here investigated. Both 8-hydroxyquinoline compounds proved effective in protecting worms from cardiotoxic LC-induced functional and ultrastructural damage, providing new mechanistic information on amyloid heart disease. Notably, PBT2, acting as metal protein attenuating compound, had beneficial effects on different proteins involved in neurodegenerative diseases such as Alzheimer and Huntington diseases (15,28).

In the *C.elegans* model, PBT2 counteracted LC toxicity at a concentration seven thousand fold lower than CQ and restored pumping in the presence of LC-induced functional damage, suggesting potential for future translation to clinical use.

The effects displayed by CQ and PBT2 have been shown to derive from multiple mechanisms of action involving both their simple action as (redox silencing) chelator and their ability to act as metal chaperones to promote protective intracellular signalling by transporting metals into the cells (5). We showed that neither compound counteracted *H₂O₂*-induced pharyngeal dysfunction and mitochondrial ROS generation, indicating that their protective activity against LC toxicity was not ascribable to a direct anti-oxidant activity (1,11). Both 8-hydroxyquinoline compounds prevented the induction by LC of pharyngeal expression of HSP-16.2 and SOD-3. In addition, they promoted the nuclear translocation of DAF-16, known to have a prominent influence on disease-related lifespan changes in *C. elegans* (40,44), an effect which was not observed when NAC, exerting only an antioxidant activity, was administered.

Although these findings were demonstrated in a simple multi cellular organism, the significant evolutionary conservation of basic biological processes between *C. elegans* and
humans strongly suggests that metal-induced oxidative stress, already reported to be linked
with some neurological disorders (18,55), is also a key element in cardiac AL
amyloidosis. Metal-binding compounds of moderate affinity, which can redistribute
biological metals, could be key drugs in an innovative therapeutic strategy designed to
target directly the mechanisms of cardiac LC toxicity. In this aspect, PBT2 is particularly
promising since it was administered for 26 weeks to Huntington' patients in the absence of
side effects (26). Cardiac involvement in AL amyloidosis is staged using the Mayo Clinic
system (14). Patients with advanced cardiac damage (stage III) can be further distinguished in
two subgroups, stage IIIa and IIIb, if they present with NT-proBNP below or above 8500
ng/L, respectively. The survival of stage IIIb patients, who represents approximately 25% of
newly diagnosed patients, is a matter of few weeks, using current therapies. Stage IIIb patients
represent an unmet need. It has been recently reported, in a case-matched study, that the
addition of doxycycline to chemotherapy can significantly reduce the early mortality in
patients with stage IIIa, but it is ineffective in patients with stage IIIb (53). The synergic
combination of PBT2 with TETRA offers a new therapeutic tool for AL patients with
cardiac damage, particularly for stage IIIb patients.
1. **INNOVATION**

2. Redox-active transition metals play a key role in driving the ROS-mediated toxic effects of LC in cardiac AL amyloidosis. Although these evidences are obtained in a simple multicellular organism, their relevance is supported by the evolutionary conservation of basic biological processes between *C. elegans* and humans and the similar subcellular alterations caused by cardiac LC in human heart and worms. Metal-binding 8-hydroxyquinoline compounds permanently blocked the ROS production and prevented the cardiotoxic effects of LC. This novel conceptual advance paves the way for new pharmacological strategies aimed at not only counteracting but totally inhibiting the vicious cycle of redox damage.
MATERIALS AND METHODS

Human samples

Urine, bone marrow plasma cells and endomyocardial biopsies were obtained from patients during routine diagnostic procedures. Acquisition, storage and use of biological samples for research purposes were approved by the Institutional Review Board. Written informed consent was received from participants prior to inclusion in the study. The presence of tissue amyloid deposits and amyloid organ involvement were defined according to the International Consensus Panel Criteria (19). LC cardiotoxicity was evaluated on the basis of clinical, echocardiography and biochemical parameters. Non amyloidogenic LC from multiple myeloma (MM) patients were used as controls. All LC included in the study were λ isotype, which represent ~75% of amyloidogenic LC. Human monoclonal LC were isolated from urine (Bence Jones, BJ) and by production, as recombinant proteins (42,46) Overall, seven LC were obtained (6 BJ and 1 recombinant) (Supplementary Table S1). Endomyocardial biopsies from three AL patients with advanced cardiac dysfunction and from one subject with primary dilated cardiomyopathy (Supplementary Table S3), used as control for disease and severity of heart dysfunction, were analyzed.

Hydrogen peroxide determination

Cardiotoxic H7-BJ and myeloma MM2-BJ proteins (1 mg/mL, corresponding to 45 µM) in 10 mM phosphate buffered saline (PBS) (pH 7.4) were incubated in the presence or absence of Chelex 100 chelating resin (5 mg/mL, Bio-Rad Laboratories, Munchen, Germany) for 1 h at 4°C with shaking according to manufacturer’s instructions. PBS and bidistilled water
were incubated with Chelex, in the same conditions. Samples were centrifuged at 8700 g x 5 min at 4°C, the supernatants were collected and the protein content was determined (Bio-Rad Laboratories GmbH, München, Germany). Different times after incubation, 2 µL of solutions were put into a 96-well black plate, diluted 1:100 (vol/vol) with 10 mM PBS, pH 7.4, and the amount of H₂O₂ generated was determined by using the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular probes, Life Technologies, Thermo Fisher, Milan, Italy, Catalog no. A22188). To determine the optimal amount of sample for the analysis, from a 20 mM H₂O₂ working solution in 1X Reaction Buffer (prepared just before use and used within a few hours of preparation) a standard curve from 0 to 10 µM was prepared. In addition, 10 µM H₂O₂ solution in 1X Reaction Buffer was used as positive control and 1X Reaction Buffer without H₂O₂ was employed as negative control. Additional controls included 10 mM PBS (pH 7.4) and bidistilled water, incubated or not with Chelex.

Experiments were done with proteins previously incubated with 50 µM CuCl₂, 50 µM ZnCl₂, 50 µM FeCl₂, 25 µM CQ (dissolved in DMSO at 250 µM, Sigma Aldrich, MO, USA) or 2 nM PBT2 (dissolved in absolute ethanol at 250 µM, Prana Biotechnology Ltd, Parkville, Australia). CuCl₂, ZnCl₂, FeCl₂, CQ and PBT2 were used as controls. The role of thiols was considered by performing experiments with proteins previously incubated with iodoacetamide (1:10 molar ratio) for 3 h at 20°C (54). Iodoacetamide alone was used as control.

EPR studies

EPR spectroscopy studies were performed as already described (13). Sixty µM of cardiotoxic (H7-BJ) and myeloma (MM2-BJ, MM7-BJ) proteins, in 10 mM PBS, pH 7.4, were incubated with or without 30µM Cu²⁺. Detection of oxygen radical species by EPR
spectroscopy was evaluated by adding the spin-trap 5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide (DEPMPO, Enzo Life Sciences, Roma, Italy), with a 1000-fold molar excess, as already described (13), on an ESP300 CW-X band spectrometer (Bruker, Milan, Italy). The EPR signal intensity was assessed based on the peak-to-trough height of a reference line in the spectrum.

C. elegans studies

Bristol N2 strain, transgenic CL2070, CF1553 and TJ356 were obtained from the Caenorhabditis elegans Genetic Center (CGC, University of Minnesota, Minneapolis, MN, USA) and propagated at 20°C on solid Nematode Growth Medium (NGM) seeded with E. coli OP50 (CGC) for food. The effect of LC on pharyngeal behavior was evaluated as already described (13). Briefly, worms were incubated with 100 μg/mL LC (100 worms/100 μL) in 10 mM PBS, pH 7.4. Control worms were incubated with 10 mM PBS, pH 7.4 (Vehicle) only. After 2 h of incubation on orbital shaking, worms were transferred onto NGM plates seeded with OP50 E. coli. The pharyngeal pumping rate, measured by counting the number of times the terminal bulb of the pharynx contracted over a 1-minute interval, was scored 20 h later. Experiments were also performed by feeding worms for 2 h with 100 μg/mL of LC alone or with: 5 mM NAC (Sigma-Aldrich) or 20-50 μM TETRA (Sigma-Aldrich) in 10 mM PBS, pH 7.4; 5 mg/mL Chelex in 10 mM PBS, pH 7.4; 10 μM ethylenediaminetetraacetic acid (EDTA, Sigma Aldrich) in metal free water (Sigma Aldrich), 0-25 μM CQ or 0-25 nM PBT2 dissolved as described above; 50-100 μM copper (from CuCl₂) in metal free water; 50-100 μM zinc (from ZnCl₂) in metal free water, 50-100 μM iron (from FeCl₂) in metal free water. These doses of copper, zinc and iron, which are well above the physiological levels in humans, are compatible
with viability of the worms and may be representative of biological relevant concentration in *C. elegans* (7,45). Higher doses of Chelex, EDTA, CQ and PBT2 were proved toxic for nematodes (22,27). The effect of 1 mM H$_2$O$_2$ for 30 min, alone or together with 5 mg/mL Chelex, 25 μM CQ or 2 nM PBT2, was also investigated. One mM H$_2$O$_2$, cardiotoxic and myeloma LC (100 μg/ml) were diluted in 50 mM phosphate buffer, pH 7.0, and incubated with 100 U/mL catalase (Sigma Aldrich) for 15 min at room temperature in dark conditions. Nematodes were incubated with these solutions (100 worms/100 μL) for 30 min on orbital shaking in dark conditions and then transferred onto NGM plates seeded with OP50 *E. coli*. The pharyngeal pumping rate was measured 2 h later.

To investigate the combined effect of TETRA and metal-binding compounds, nematodes (100 worms/100 μL) were fed 100 μg/mL H7 alone for 2 h and then treated for 30 min with CQ (25 μM), PBT2 (0.5-2 nM), 20 μM TETRA alone or together with 0.5-2 nM PBT2. Worms were then transferred onto fresh NGM plates seeded with *E. coli* in the presence of the same drug concentration and the pharyngeal pumping rate was scored after 20 h. Worms were also exposed to the drugs alone or to vehicle in the same experimental conditions.

For lifespan experiments N2 worms (100 worms/100 μL), at L3 larval stage, were fed for 2 h 100 μg/mL of H7 alone or with 25 μM CQ or 2 nM PBT2 (13). Freshly 2 nM PBT2 was daily added to PBT2-treated worms. The number of live worms was determined for each consecutive day until all worms were dead.

**Mitochondrial production of ROS**

The effect of LC on mitochondrial oxidant burden was evaluated by feeding worms with MitoSOX Red (Molecular Probes, Italy) as already described (13).
Mitochondrial membrane potential

N2 worms were incubated with 100 µg/mL of LC (100 worms/100 µL) in 10 mM PBS (pH 7.4). Negative control worms were incubated with 10 mM PBS (pH 7.4) (vehicle) only and positive control worms were incubated with 10 mM H$_2$O$_2$. After 2 h incubation with orbital shaking, worms were plated on NGM plates seeded with OP50 E. coli pre-treated for 2 h with 20 µM tetramethylrhodamine, methyl ester (TMRM, Molecular Probes, Thermo Fisher Scientific). After 24 h, worms were immobilised with 20 mM levamisole and immediately used for microscopic examination with an inverted fluorescent microscope (IX-71 Olympus) equipped with a CCD camera. Images of the pharynges were taken at 40 X magnification with a TRITC filter set (Olympus).

Transmission electron microscopy (TEM) analysis

Worms fed cardiotoxic LC or myeloma alone or with drugs, as described before, were picked, washed in 10 mM PBS, pH 7.4, and fixed with 2% glutaraldehyde in 0.12 M phosphate buffer, pH 7.4. Worms were cut open at the level of second bulb of the pharynx, to improve access of the fixative. After post-fixation at room temperature overnight, samples were incubated in a solution of 1% OsO$_4$ and 1.5% ferrocyanide in 0.12 M cacodylate buffer (ferrocyanide-reduced OsO$_4$) at room temperature for 1 h, then 0.3% thiocarbohydrazide in water for 5 min, and finally 2% OsO$_4$ in 0.12 M cacodylate buffer for 1 h. C. elegans pharynx was then placed into 2% agarose gel and small cubes were cut and dehydrated in graded series of ethanol for 10 min each, cleared in propylene oxide and embedded in Epoxy medium (Epon 812 Fluka) and polymerized at 60°C for 72 h. From each sample, one semithin (1 µm) section was cut with a
Leica EM UC6 ultramicrotome and mounted on glass slides for light microscopic inspection.

Ultrathin (60-80 nm thick) sections of areas of interest were obtained, counterstained with uranyl acetate and lead citrate, and examined with an Energy Filter TEM (ZEISS LIBRA® 120) equipped with a YAG scintillator slow scan CCD camera.

Specimens of human myocardial tissue were fixed with 2.5% glutaraldehyde in 0.2 M cacodylate buffer, pH 7.3, for 2 h, and post-fixed in 1% OsO₄ in the same buffer. They were dehydrated in a graded series of ethyl alcohols and embedded in epoxy resin. Ultrathin sections (60-80 nm thick) were cut, mounted on nickel grids and stained with 5% uranyl acetate and lead citrate. A minimum of 5 sections for each patient were observed with a Philips CM12 TEM.

Sections were then processed for post-embedding immunogold (21). Enzymatic predigestion (0.05% trypsin in 0.05 M Tris buffer with 0.05% CaCl₂, 37°C, 15 min) to unmask antigenic epitopes was performed. Sections were rinsed in 0.05 M Tris/HCl buffer, pH 7.3, incubated with either 1:20 normal goat serum or 1% egg albumin for 15 min at room temperature. The sections were incubated overnight at 4°C with polyclonal anti-λ LC antibody (dilution 1:50, Dako, Agilent Technologies, CA, USA), then incubated for 1 h at room temperature with protein-A (dilution 1:20) conjugated to 15 nm colloidal gold particles (British Biocell International, UK). Specificity of immunoreactions was verified using either normal goat serum or egg albumin as primary antibody.

**DAF-16 translocation assay and pharyngeal expression of heat shock protein (HSP)-16 and superoxidizedismutase (SOD)-3**

DAF-16::green fluorescent protein (GFP) nuclear translocation was evaluated in TJ356 nematodes. Pharyngeal expression of heat shock protein (HSP)-16.2::GFP and superoxide...
dismutase (SOD)-3::GFP was determined in CL2070 and CF1553 worms, respectively.

Nematodes fed H7-BJ or MM2-BJ in the absence or presence of CQ, PBT2, TETRA and NAC as already described. Control worms were incubated with 10 mM PBS, pH 7.4, (vehicle) or drugs alone. After 2-20 h, nematodes were paralyzed by adding 20 mM levamisole, centrifuged at 2000 x g for 5 min and fixed in 4% paraformaldehyde in 10 mM PBS, pH 7.4, for 24 h at 4°C. Organisms were scored as positive for nuclear localization of DAF-16::GFP when green foci were observed throughout the entire body from head to tail and as cytosolic when DAF-16::GFP was diffuse. The number of worms with each level of translocation were counted (n=100 worms/condition). To prove the nuclear localization of activated DAF-16::GFP, nuclear counterstaining was performed with 2′-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5′-bi-1H-benzimidazole trihydrochloride trihydrate (Hoechst 33342 Hoechst, Thermo Fisher Scientific). Nematodes were fed 100 µg/mL of cardiotoxic H7-BJ LC (100 worms/100 µL) in 10 mM PBS (pH 7.4).

Control worms were incubated with 10 mM PBS (pH 7.4) (vehicle) only. After 2 h incubation with orbital shaking, worms were paralysed by adding 20 mM levamisole, centrifuged at 2000 x g for 3 min and fixed in 4% paraformaldehyde in 10 mM PBS (pH 7.4) for 2 h at 4°C. Nematodes were centrifuged at 2000 x g for 3 min, washed twice with 10 mM PBS (pH 7.4) and resuspended in 0.5 mL of 125 mM Tris-HCl solution (pH 7.4) containing 1% Triton X-100 and 5% β-mercaptoethanol. After overnight incubation at 4°C, worms were washed with 10 mM PBS (pH 7.4) and incubated at room temperature for 30 min in 0.5 mL of Hoechst (1 mg/mL in 10 mM PBS, pH 7.4). Nuclear translocation of DAF-16 and Hoechst was visualised at 40 X magnification with a GFP and a UV filter, respectively, with an inverted fluorescent microscope (IX-71 Olympus) equipped.
1. Images for HSP-16.2::GFP and SOD-3::GFP expression in the pharynx of worms were acquired using the same exposure settings. Average pixel intensity values were calculated by sampling images of different animals. Mean pixel intensity for each experimental group was calculated using Cell-F software (Olympus).

6. **Statistical analysis**

8. The data were analyzed using GraphPad Prism 6.0 software (CA, USA) by an independent Student’s t-test, one-way and two-way ANOVA and Bonferroni’s *post hoc* test. The values of IC$_{50}$ and median survival were determined using Prism version 6.0 for Windows (GraphPad Software, CA, USA). A *p* value $< 0.05$ was considered statistically significant.
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AUTHOR DISCLOSURE STATEMENT

RAC is a paid consultant to and a shareholder in Prana Biotechnology Ltd. The other authors have no competing interests.
**LIST OF ABBREVIATIONS**

1. AL = Immunoglobulin light chains amyloidosis  
2. BJ = Bence Jones  
3. CD = Circular Dichroism  
4. CQ = 5-chloro-7-iodo-quinolin-8-ol  
5. DEPMPO = 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide  
6. EPR = Electron paramagnetic resonance  
7. F = Female  
8. FI = Fluorescence Intensity  
9. FLC = Free Light Chains  
10. FOXO = Forkhead transcription factors  
11. GFP = Green fluorescent protein  
12. H = Heart  
13. Hoechst = 2'-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole trihydrochloride trihydrate  
14. HSP = Heat shock protein  
15. I/IGF-1 = insulin/insulin growth factor-1  
16. LC = Immunoglobulin light chains  
17. M = Male  
18. MM = Multiple myeloma  
19. NAC = N-acetyl-cysteine  
20. NGM = Nematode Growth medium  
21. PBS = Sodium phosphate buffer
1. ROS= Reactive oxygen species
2. SOD= Superoxide dismutase
3. TEM= Transmission electron microscopy
4. TETRA= Tetracycline hydrochloride
5. TMRM= Tetramethylrhodamine, methyl ester
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FIGURE LEGENDS

FIG. 1. Effect of metal ions on the ability of LC to generate ROS and affect pharyngeal pumping in worms. (A) H$_2$O$_2$ produced by cardiotoxic LC (H7-BJ) and myeloma (MM2-BJ) incubated 2 h with or without 5 mg/mL Chelex. Mean ± SE of fluorescence intensity (FI), n=6, **p<0.01 vs. cardiotoxic LC and °° p<0.01 vs. myeloma, one-way ANOVA and Bonferroni’s post hoc test. (B) Worms were fed for 2 h with 100 µg/mL cardiotoxic LC (H6-BJ, H7-BJ, H7-r, H18-BJ), myeloma proteins (MM2-BJ, MM4-BJ, MM7-BJ) with or without 5 mg/mL Chelex or 10 µM EDTA. Control worms were incubated with 10 mM PBS, pH 7.4 (Vehicle) only (dotted line). The mean ± 95% CI of pumps/min was calculated (horizontal line). Each dot is the mean of pumps/min for each protein (3 independent assays, n= 30 worms/assay). **p<0.01 vs. Vehicle, °°p<0.01 vs. cardiotoxic LC, one-way ANOVA and Bonferroni’s post hoc test. (C) H$_2$O$_2$ produced by 45 µM cardiotoxic LC and myeloma treated with Chelex and incubated 2 h with 50 µM CuCl$_2$, ZnCl$_2$ or FeCl$_2$. Control samples were incubated with Chelex-treated 10 mM PBS, pH 7.4. Mean ±SE of FI, n=12, **p<0.01 vs. cardiotoxic LC incubated with Chelex-treated PBS, pH 7.4, one-way ANOVA and Bonferroni’s post hoc test. (D) Worms were fed for 2 h with 100 µg/mL cardiotoxic LC (H7-BJ), myeloma (MM2-BJ) with or without 50 µM CuCl$_2$, ZnCl$_2$ or FeCl$_2$. Control worms were incubated with 10 mM PBS, pH 7.4 (Vehicle) only or 50 µM CuCl$_2$, ZnCl$_2$ and FeCl$_2$. Pumping rate as mean pumps/min ± SE (n= 20 worms/assay, 3 assays). **p<0.01 vs. vehicle, one-way ANOVA and Bonferroni’s post hoc test. °°p<0.0001 vs. cardiotoxic LC, two-way ANOVA and Bonferroni’s post hoc test. (E) H$_2$O$_2$ produced by cardiotoxic LC (H7-BJ) and myeloma (MM2-BJ) previously treated for 3 h at 20°C with iodoacetamide and then

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incubated 2 h with 50 µM CuCl₂. Mean ± SE of FI, n=9, **p<0.01 vs. cardiotoxic LC not treated with iodoacetamide and °° p<0.01 vs cardiotoxic LC treated with iodoacetamide, one-way ANOVA and Bonferroni’s post hoc test.

FIG. 2. Effect of metal-binding compounds CQ and PBT2 on LC-induced H₂O₂ production and oxidative damage. (A) Dose-response effect of CQ and PBT2 on LC-induced pharyngeal dysfunction. Worms were fed for 2 h with 100 µg/mL cardiotoxic LC in the absence or presence of 0-25 µM CQ or 0-25 nM PBT2. Control worms received vehicle alone (dotted line). Each value is the mean ± SE, n=30. IC₅₀ ± SD are reported, p<0.01 Student’s t test. (B) H₂O₂ produced by cardiotoxic LC (H7-BJ) incubated 2 h with or without 2 nM PBT2 or 25 µM CQ. Mean ± SE of FI, n=6, **p<0.01 vs. cardiotoxic LC, one-way ANOVA and Bonferroni’s post hoc test. (C-D) Worms were fed for 2 h with 100 µg/mL cardiotoxic LC (H6-BJ, H7-BJ, H7-r, H18-BJ) with or without 25 µM CQ or 2 nM PBT2. H₂O₂ (1 mM) was administered for 30 min with or without the drugs. Control worms received vehicle alone (dotted line). (C) The mean ± 95% CI of pumps/min was calculated (horizontal line). Each dot is the mean of pumps/min for each protein (3 independent assays, n=30 worms/assay). **p<0.01 vs. Vehicle, °°p<0.01 vs. cardiotoxic LC, one-way ANOVA and Bonferroni’s post hoc test. (D) Images obtained from the overlay of a contrast phase and MitoSOX fluorescence (arrows). Scale bar 50 µm. (E) Kaplan-Meier survival curves, n=30 worms/group, 3 independent experiments.
FIG. 3. ROS-induced cardiotoxic LC severely disrupt C. elegans pharyngeal ultrastructure. (A-G) Representative images of worm’s pharynx obtained from the ultrastructural analysis by TEM in C. elegans fed for 2 h with: (A) Vehicle, (B) myeloma protein (MM2-BJ), (C) cardiotoxic LC (H7-BJ) alone or with (D) 25 µM CQ, (E) 2 nM PBT2, (F) 50 µM TETRA or (G) 5 mM NAC. Images showed two pharyngeal muscles (pm) with their mitochondria (arrowheads) separated by a marginal cell (mc) and its mitochondria (arrows), placed at the corner of the pharyngeal channel (ch). Scale bar, 500 nm. Pharyngeal muscles of worms fed cardiotoxic LC resulted in a damage to mitochondria which exhibited a clustering pattern and irregular shape, swelling and massive disruption of the internal components (i.e. cristae). Marginal cells, which contain many mitochondria due to their active role in contractile motor function, were seriously compromised and myofilaments connected to the marginal cells, which were perfectly aligned in vehicle-fed worms, were deranged.

FIG. 4. Mitochondrial damage in heart muscle tissue of cardiac AL patients. Ultrastructural details from representative TEM images of endomyocardial biopsies from (A–C) severe cardiac amyloid AL patients and (D) a patient affected by dilatative cardiomyopathy. Although myocardial fibers (mf) are relatively well preserved in AL patients, most mitochondria (white arrows) show remarkable alterations with enlarged size and disruption (A) or total loss of cristae (B–C). LC were identified by post embedding immunogold staining with 15 nm gold-conjugated protein A (black arrows) in the interstitium and along the basement membrane of a myocardial fiber. (D) The myocardium of a patient with non-amyloid cardiomyopathy shows well preserved mitochondria (white.
arrows) and glycogen deposits (g). To better highlight the differences in damage, individual mitochondria in the red insets are shown in Supplementary Fig. S7. Uranyl acetate, lead citrate. Scale bar, 1 µm.

**FIG. 5. Metal ions drive the ability of cardiotoxic LC to promote DAF-16 translocation from cytoplasm to nucleus in TJ356 transgenic worms.** (A-B) Image of DAF-16::GFP expression in (A) control vehicle-fed and (B) cardiotoxic LC-fed worms (100 µg/mL H7-BJ for 2 h). (C-D) The subcellular distribution of DAF-16 expression in worms fed 2 h: vehicle, 100 µg/mL cardiotoxic LC with or without 25 µM CQ, 2 nM PBT2, 50 µM TETRA or 5 mM NAC. According to DAF-16 localization worms were divided into two phenotypes including “cytosolic” and “nuclear”. The percentage of DAF-16 localization in respect to vehicle fed worms was calculated based on 3 experiments, n=100. Mean ±SE. ** p<0.01 vs. vehicle, ° p<0.05 and °° p<0.01 vs. cardiotoxic LC, one-way ANOVA and Bonferroni’s post hoc test.

**FIG. 6. Metal ions drive the ability of cardiotoxic LC to induce the pharyngeal expression of HSP-16.2 and SOD-3.** (A-D) Transgenic worms were fed for 2 h with: Vehicle (i, 10 mM PBS, pH 7.4), 100 µg/mL myeloma (ii, MM2-BJ), 100 µg/mL cardiotoxic LC (iii, H7-BJ), cardiotoxic LC + 25 µM CQ (iv) or 2 nM PBT2 (v, cardiotoxic LC+ PBT2). (A) Images of the HSP-16.2 expression as overlays of GFP-fluorescence and light microscopy in CL2070 transgenic worms. Scale bar, 50 µm. (C) Images of SOD-3 expression as GFP-fluorescence (arrows) in CF1553 transgenic worms. Scale bar, 50 µm. Quantified GFP intensity in (B) CL2070 and (D) CF1553 worms in response to
treatments. Fluorescence intensity in each group was calculated as mean grey value ± SE based on 3 experiments, n= 25. ** p<0.01 vs. Vehicle and °p<0.05 and °° p<0.01 vs. cardiotoxic LC, one-way ANOVA and Bonferroni’s post hoc test.

FIG. 7. Synergistic beneficial effect of PBT2 and TETRA. (A-B) Pharyngeal performance of worms fed 100 µg/mL cardiotoxic LC (H7 SBJ) for 2 h and then treated for 30 min with (A) 25 µM CQ, 20 µM TETRA or 2 nM PBT2 or with (B) 0.5-2 nM PBT2 alone or together with 20 µM TETRA. Control worms fed vehicle alone. **p<0.001, *p<0.005 vs. vehicle, °° p<0.001 vs. cardiotoxic LC, one-way ANOVA and Bonferroni’s post hoc test. §§ p<0.01 significant interaction vs. worms fed cardiotoxic LC+ 0.5 nM PBT2, two-way ANOVA and Bonferroni’s post hoc test.

FIG. 8. Proposed model for metal ions involvement in the mechanism underlying the LC-induced toxicity. Based on our current knowledge, redox-active transition metals, particularly copper, drive the ability of cardiotoxic LC to produce ROS in vivo. The excessive production of ROS can directly target the pharyngeal cells, damaging the organelle functions and the ultrastructure, particularly at the mitochondrial level. ROS can be also produced as result of the mitochondria dysfunction requiring copper and iron for the activation of the enzymes involved in the oxidative phosphorylation pathway. Intracellular signaling events aimed at limiting and repairing the stress-induced damage are activated. Mitochondria reacts to ROS by inducing the expression of the scavenger protein SOD-3. In addition, the chaperone HSP-16.2, an αB-crystallin-related protein, is activated as well as the nuclear translocation of the FOXO/DAF-16 transcription factor. This last one can trigger a secondary
ROS-induced cellular response by inducing the transcription of stress-responsive genes, including $hsp-16.2$ and $sod-3$, and controlling longevity.
FIG. 1. Effect of metal ions on the ability of LC to generate ROS and affect pharyngeal pumping in worms. (A) H$_2$O$_2$ produced by cardiotoxic LC (H7-BJ) and myeloma (MM2-BJ) incubated 2 h with or without 5 mg/mL Chelex. Mean ± SE of fluorescence intensity (FI), n=6, **p<0.01 vs. cardiotoxic LC and °° p<0.01 vs. myeloma, one-way ANOVA and Bonferroni’s post hoc test. (B) Worms were fed for 2 h with 100 µg/mL cardiotoxic LC (H6-BJ, H7-BJ, H7-r, H18-BJ), myeloma proteins (MM2-BJ, MM4-BJ, MM7-BJ) with or without 5 mg/mL Chelex or 10 µM EDTA. Control worms were incubated with 10 mM PBS, pH 7.4 (Vehicle) only (dotted line). The mean ± 95% CI of pumps/min was calculated (horizontal line). Each dot is the mean of pumps/min for each protein (3 independent assays, n= 30 worms/assay). **p<0.01 vs. Vehicle, °°p<0.01 vs. cardiotoxic LC, one-way ANOVA and Bonferroni’s post hoc test. (C) H$_2$O$_2$ produced by 45 µM cardiotoxic LC and myeloma treated with Chelex and incubated 2 h with 50 µM CuCl$_2$, ZnCl$_2$ or FeCl$_2$. Control samples were incubated with Chelex-treated 10 mM PBS, pH 7.4. Mean ±SE of FI, n=12, **p<0.01 vs. cardiotoxic LC incubated with Chelex-treated PBS, pH 7.4, °° p<0.01 vs. myeloma LC + Chelex-treated PBS, pH 7.4, one-way ANOVA and Bonferroni’s post hoc test. (D) Worms were fed for 2 h with 100 µg/mL cardiotoxic LC.
(H7-BJ), myeloma (MM2-BJ) with or without 50 µM CuCl$_2$, ZnCl$_2$ or FeCl$_2$. Control worms were incubated with 10 mM PBS, pH 7.4 (Vehicle) only or 50 µM CuCl$_2$, ZnCl$_2$ and FeCl$_2$. Pumping rate as mean pumps/min ± SE (n= 20 worms/assay, 3 assays). **p<0.01 vs. vehicle, one-way ANOVA and Bonferroni’s post hoc test. °°p<0.0001 vs. cardiotoxic LC, two-way ANOVA and Bonferroni’s post hoc test. (E) H$_2$O$_2$ produced by cardiotoxic LC (H7-BJ) and myeloma (MM2-BJ) previously treated for 3 h at 20°C with iodoacetamide and then incubated 2 h with 50 µM CuCl$_2$. Mean ± SE of FI, n=9, **p<0.01 vs. cardiotoxic LC not treated with iodoacetamide and °° p<0.01 vs. cardiotoxic LC treated with iodoacetamide, one-way ANOVA and Bonferroni’s post hoc test.
FIG. 2. Effect of metal-binding compounds CQ and PBT2 on LC-induced H$_2$O$_2$ production and oxidative damage. (A) Dose-response effect of CQ and PBT2 on LC-induced pharyngeal dysfunction. Worms were fed for 2 h with 100 µg/mL cardiotoxic LC in the absence or presence of 0-25 µM CQ or 0-25 nM PBT2. Control worms received vehicle alone (dotted line). Each value is the mean ± SE, n=30. IC$_{50}$ ± SD are reported, p<0.01 Student’s t test. (B) H$_2$O$_2$ produced by cardiotoxic LC (H7-B1) incubated 2 h with or without 2 nM PBT2 or 25 µM CQ. Mean ± SE of FI, n=6, **p<0.01 vs. cardiotoxic LC, one-way ANOVA and Bonferroni’s post hoc test. (C-D) Worms were fed for 2 h with 100 µg/mL cardiotoxic LC (H6-BJ, H7-BJ, H7-r, H18-BJ) with or without 25 µM CQ or 2 nM PBT2. H$_2$O$_2$ (1 mM) was administered for 30 min with or without the drugs. Control worms received vehicle alone (dotted line). (C) The mean ± 95% CI of pumps/min was calculated (horizontal line). Each dot is the mean of pumps/min for each protein (3 independent assays, n=30 worms/assay). **p<0.01 vs. Vehicle, °°p<0.01 vs. cardiotoxic LC, one-way ANOVA and Bonferroni’s post hoc test. (D) Images obtained from the overlay of a contrast phase and MitoSOX fluorescence (arrows). Scale bar 50 µm. (E) Kaplan-Meier survival curves, n=30 worms/group, 3 independent experiments.

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254x190mm (300 x 300 DPI)
FIG. 3. ROS-induced cardiotoxic LC severely disrupt *C. elegans* pharyngeal ultrastructure. (A-G)

Representative images of worm’s pharynx obtained from the ultrastructural analysis by TEM in *C. elegans* fed for 2 h with: (A) Vehicle, (B) myeloma protein (MM2-BJ), (C) cardiotoxic LC (H7-BJ) alone or with (D) 25 µM CQ, (E) 2 nM PBT2, (F) 50 µM TETRA or (G) 5 mM NAC. Images showed two pharyngeal muscles (pm) with their mitochondria (arrowheads) separated by a marginal cell (mc) and its mitochondria (arrows), placed at the corner of the pharyngeal channel (ch). Scale bar, 500 nm. Pharyngeal muscles of worms fed cardiotoxic LC resulted in a damage to mitochondria which exhibited a clustering pattern and irregular shape, swelling and massive disruption of the internal components (i.e. cristae). Marginal cells, which contain many mitochondria due to their active role in contractile motor function, were seriously compromised and myofilaments connected to the marginal cells, which were perfectly aligned in vehicle-fed worms, were deranged.

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252x124mm (300 x 300 DPI)
FIG. 4. Mitochondrial damage in heart muscle tissue of cardiac AL patients. Ultrastructural details from representative TEM images of endomyocardial biopsies from (A-C) severe cardiac amyloid AL patients and (D) a patient affected by dilatative cardiomyopathy. Although myocardial fibers (mf) are relatively well preserved in AL patients, most mitochondria (white arrows) show remarkable alterations with enlarged size and disruption (A) or total loss of cristae (B-C). LC were identified by post embedding immunogold staining with 15 nm gold-conjugated protein A (black arrows) in the interstitium and along the basement membrane of a myocardial fiber. (D) The myocardium of a patient with non-amyloid cardiomyopathy shows well preserved mitochondria (white arrows) and glycogen deposits (g). To better highlight the differences in damage, individual mitochondria in the red insets are shown in Supplementary Fig. S7. Uranyl acetate, lead citrate. Scale bar, 1 µm.
FIG. 5. Metal ions drive the ability of cardiotoxic LC to promote DAF-16 translocation from cytoplasm to nucleus in TJ356 transgenic worms. (A-B) Image of DAF-16::GFP expression in (A) control vehicle-fed and (B) cardiotoxic LC-fed worms (100 µg/mL H7-BJ for 2 h). (C-D) The subcellular distribution of DAF-16 expression in worms fed 2 h: vehicle, 100 µg/mL cardiotoxic LC with or without 25 µM CQ, 2 nM PBT2, 50 µM TETRA or 5 mM NAC. According to DAF-16 localization worms were divided into two phenotypes including “cytosolic” and “nuclear”. The percentage of DAF-16 localization in respect to vehicle fed worms was calculated based on 3 experiments, n=100. Mean ±SE. ** p<0.01 vs. vehicle, ° p<0.05 and °° p<0.01 vs. cardiotoxic LC, one-way ANOVA and Bonferroni’s post hoc test.
FIG. 6. Metal ions drive the ability of cardiotoxic LC to induce the pharyngeal expression of HSP-16.2 and SOD-3. (A-D) Transgenic worms were fed for 2 h with: Vehicle (i, 10 mM PBS, pH 7.4), 100 µg/mL myeloma (ii, MM2-BJ), 100 µg/mL cardiotoxic LC (iii, H7-BJ), cardiotoxic LC + 25 µM CQ (iv) or 2 nM PBT2 (v, cardiotoxic LC+ PBT2). (A) Images of the HSP-16.2 expression as overlays of GFP-fluorescence and light microscopy in CL2070 transgenic worms. Scale bar, 50 µm. (C) Images of SOD-3 expression as GFP-fluorescence (arrows) in CF1553 transgenic worms. Scale bar, 50 µm. Quantified GFP intensity in (B) CL2070 and (D) CF1553 worms in response to treatments. Fluorescence intensity in each group was calculated as mean grey value ± SE based on 3 experiments, n= 25. ** p<0.01 vs. Vehicle and °° p<0.01 vs. cardiotoxic LC, one-way ANOVA and Bonferroni’s post hoc test.
FIG. 7. Synergistic beneficial effect of PBT2 and TETRA. (A-B) Pharyngeal performance of worms fed 100 µg/mL cardiotoxic LC (H7-BJ) for 2 h and then treated for 30 min with (A) 25 µM CQ, 20 µM TETRA or 2 nM PBT2 or with (B) 0.5-2 nM PBT2 alone or together with 20 µM TETRA. Control worms fed vehicle alone. **p<0.001, *p<0.005 vs. vehicle, °° p<0.001 vs. cardiotoxic LC, one-way ANOVA and Bonferroni’s post hoc test. §§ p<0.01 significant interaction vs. worms fed cardiotoxic LC+ 0.5 nM PBT2, two-way ANOVA and Bonferroni’s post hoc test.
FIG. 8. Proposed model for metal ions involvement in the mechanism underlying the LC-induced toxicity. Based on our current knowledge, redox-active transition metals, particularly copper, drive the ability of CardiotoxicLC to produce ROS in vivo. The excessive production of ROS can directly target the pharyngeal cells, damaging the organelle functions and the ultrastructure, particularly at the mitochondrial level. ROS can be also produced as result of the mitochondria dysfunction requiring copper and iron for the activation of the enzymes involved in the oxidative phosphorylation pathway. Intracellular signaling events aimed at limiting and repairing the stress-induced damage are activated. Mitochondria reacts to ROS by inducing the expression of the scavenger protein SOD-3. In addition, the chaperone HSP-16.2, an αβ-crystallin-related protein, is activated as well as the nuclear translocation of the FOXO/DAF-16 transcription factor. This last one can trigger a secondary ROS-induced cellular response by inducing the transcription of stress-responsive genes, including hsp-16.2 and sod-3, and controlling longevity.
SUPPLEMENTARY INFORMATION

Original Research Communication

Cardiac light chain amyloidosis: The role of metal ions in oxidative stress and mitochondrial damage

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Abbreviated Title: Metal ions drive cardiac light chain toxicity

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**SUPPLEMENTARY DATA**

**Supplementary Table S1.** Clinical and biochemical characteristics of patients at diagnosis of immunoglobulin light chain amyloidosis or multiple myeloma.

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<th>Code</th>
<th>Gender, Age</th>
<th>Cardiac stage°</th>
<th>Diagnosis</th>
<th>Organ involved °°</th>
<th>Recombinant</th>
<th>BJ</th>
<th>Germline gene</th>
<th>Deduced MW (kDa/pI)</th>
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<th>Proteinuria (g/24h)</th>
<th>Creatinine (mg/dL)</th>
<th>NT-proBNP (ng/L)</th>
<th>cTnI (ng/mL)</th>
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AL, Immunoglobulin light chain amyloidosis; MM, multiple myeloma; M, male; F, female; °According to Gertz et al. (19); H, Heart; °°According to the International Consensus Panel criteria (19); BJ, Bence Jones; n.a., not available; pI, isoelectric point; FLC, Free Light Chains; NT-proBNP, N-terminal prohormone of Brain Natriuretic Peptide; cTnI, cardiac Troponin I; IVS, Interventricular Septum; PW, Posterior Wall; EF, Ejection Fraction. § Entirely constituted by BJ proteins. Reference ranges: serum κ/λ FLC <26.3 mg/L, κ/λ ratio 0.26-1.65; serum creatinine <1.18 mg/dL in men, <1.02 mg/dL in women; NT-proBNP (42) <332 ng/L; BNP, <50 ng/L; cTnI <0.04 ng/mL. * BNP (ng/L).
**Supplementary Table S2.** Effect of CQ and PBT2 on the pharyngeal toxicity induced by the natural Bence Jones and recombinant LC from the same cardiac amyloid patient.

<table>
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<tr>
<th>TREATMENT</th>
<th>PHARYNGEAL PUMPING (Pumps/min± SE)</th>
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<tr>
<td>Vehicle</td>
<td>233.2 ± 1.5</td>
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<td>H7-BJ</td>
<td>209.5 ± 1.6 **</td>
</tr>
<tr>
<td>H7-r</td>
<td>212.4 ± 1.0 **</td>
</tr>
<tr>
<td>H7-BJ + CQ</td>
<td>235.2 ± 1.2</td>
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<tr>
<td>H7-BJ + PBT2</td>
<td>238.0 ± 1.4</td>
</tr>
<tr>
<td>H7-r + CQ</td>
<td>237.0 ± 1.0</td>
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<tr>
<td>H7-r + PBT2</td>
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Human monoclonal amyloidogenic cardiotoxic LC obtained as natural Bence Jones (H7-BJ) and recombinant (H7-r) from the same cardiac amyloid patient, were administered to worms (100 worms/100 µL) at 100 µg/mL in 10 mM PBS, pH 7.4, in the absence or presence of 25 µM CQ or 2 nM PBT2. Control worms received vehicle alone (Vehicle). After incubation for 2 h in the absence of OP50 *E. coli*, worms were plated on NGM plates seeded with bacteria. Pharyngeal functionality evaluated 20 h after plating. Pharyngeal pumping is expressed as pumps/minute ± S.E. **p<0.01 vs. vehicle, according to one-way ANOVA followed by Bonferroni’s post hoc test.
Supplementary Table S3. Clinical and biochemical characteristics of patients suffering from immunoglobulin light chain amyloidosis with severe cardiac involvement and subjected to endomyocardial biopsy.

<table>
<thead>
<tr>
<th>Code</th>
<th>Gender, age</th>
<th>Cardiac stage°</th>
<th>Germline gene</th>
<th>Serum λ FLC (mg/L)</th>
<th>NT-proBNP (ng/L)</th>
<th>cTnI (ng/mL)</th>
<th>IVS (mm)</th>
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AL, Immunoglobulin light chain amyloidosis; F, female; M, male; °According to Gertz et al. (19); n.a., not available; FLC, Free Light Chains; NT-proBNP, N-terminal pro hormone of Brain Natriuretic Peptide; cTnI, cardiac Troponin I; IVS, Interventricular Septum; PW, Posterior Wall; EF, Ejection Fraction. Reference ranges: serum λ FLC <26.3 mg/L; NT-proBNP (42) <332 ng/L; cTnI <0.04 ng/mL.
**Supplementary FIG. S1.** Effect of metal ions chelators on the secondary structure and thermal stability of LC. (A) Far-UV circular dichroism (CD) spectra of cardiotoxic LC (H7-BJ) and myeloma (MM2-BJ) proteins treated or not with 5 mg/mL Chelex, 25 µM CQ or 2 nM PBT2.
PBT2. All samples, at protein concentration of 0.4 mg/mL in 50 mM sodium phosphate, pH 7.4, were incubated overnight at room temperature. Far-UV CD measurements were performed at 25°C in 50 mM sodium phosphate, pH 7.4, with a Jasco J-700 spectropolarimeter (Jasco Europe, Cremella, Italy) using a quartz cuvette with a path length of 1 mm. Scans were conducted from 250 to 200 nm at a speed of 100 nm/min with a spectral band width of 2 nm, a sensitivity of 20 mdegress and response time of 1 sec. The α-helical and β-sheet content was calculated with K2D, CDSSTR and CONTIN software applications CD. CD spectra represent the average of 10 scans. Data are shown as mean residue ellipticity (MRE, deg cm$^2$ dmol$^{-1}$) as function of wavelength. All spectra presented a strong negative band at 216-218 nm. Both cardiotoxic and myeloma proteins had a similar β-sheet content, consistent with the pattern of a typical immunoglobulin, which were not modified by the presence of metal ions chelating agent. (B) Melting data were recorded at 202 nm to monitor β-sheet unfolding upon sample heating from 37°C to 80°C. Analyses were performed in a 1 mm path length quartz cuvette, temperature slope of 1°C/min, band width of 2 nm, data pitch of 0.2°C and response time of 2 sec. The thermal melt value (Tm) for each condition was calculated at the midpoint of the unfolding transition. No statistical difference among the various conditions tested was observed. No significant perturbations in thermostability were observed in LC after metal ions chelation.
Supplementary FIG. S2. Effect of catalase on cardiotoxic LC-induced pharyngeal dysfunction. Worms were fed for 30 min with 100 µg/mL cardiotoxic LC (H7-BJ) or myeloma (MM2-BJ), or 1 mM hydrogen peroxide (used as positive control) previously incubated or not with 100 U/mL catalase for 15 min at room temperature in dark conditions. Control worms received 50 mM phosphate buffer, pH 7.0 (Vehicle). Pumping rate as mean pumps/min ± SE (3 independent assays, n= 30 worms/assay). **p<0.01 vs. Vehicle, °°p<0.01 vs. the corresponding hydrogen peroxide or cardiotoxic LC not treated with catalase, one-way ANOVA and Bonferroni’s post hoc test.
**Supplementary FIG. S3. Effect of Chelex and copper on LC-induced toxicity.** Worms were fed for 2 h with 100 µg/mL cardiotoxic LC (H7-BJ) or myeloma (MM2-BJ) treated or not with 5 mg/mL of Chelex and 50 µM copper. Control worms received 50 mM phosphate buffer, pH 7.4, alone (Vehicle). Pumping rate as mean pumps/min ± SE (3 independent assays, n= 30 worms/assay). **p<0.01 vs. Vehicle, °°p<0.01 vs. cardiotoxic LC, one-way ANOVA and Bonferroni’s post hoc test.
Supplementary FIG. S4. Effect of copper on cardiotoxic LC radical production. (A) Electron paramagnetic resonance (EPR) spectra of radical adducts with spin-trap 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide (DEPMPO) obtained in the presence of 60 µM cardiotoxic LC (H7-BJ), 60 µM cardiotoxic LC + 30 µM Cu²⁺, and 30 µM Cu²⁺ only. (B). Relative intensity and time-dependence of the EPR signals reported in A. The presence of Cu²⁺ ions enhances the production of radicals by the cardiotoxic protein.
Supplementary FIG. S5. Endogenous metal ions levels in *C. elegans*. Synchronous populations of N2 worms were grown on NGM plates with OP50 *E. coli* as the food source. Worms were collected at L3 larval stage with metal free water (Sigma Aldrich), pelletted by centrifugation and fed 100 µg/mL cardiototoxic (H7-BJ) or myeloma (MM2-BJ) proteins (100 worms/100 µL, a total of 10,000 worms) in metal free water, alone or with 25 µM CQ or 2 nM PBT2. Control worms were incubated with metal free water alone (Vehicle). After 2 h of incubation on orbital shaking, worms were collected with metal free water, pelleted by centrifugation and washed in metal free water three times over a total of 30 min to clear gut
content. The worm pellets were dried for 48 h at 60°C and then digested in 6 mL of 65% HNO₃ and 2 mL of 30% H₂O₂. Samples were diluted to a final volume of 10 mL metal free water for analysis by inductively coupled plasma-atomic emission spectrometry (Varian Simul Vista MPX Radial ICP-OES, Varian Inc.). Data are expressed as the (A) copper and (B) iron and zinc percentage of control from three biological replicates ± SD. **p<0.01 vs. Vehicle, °°p<0.01 vs. cardiotoxic LC, one-way ANOVA and Bonferroni’s post hoc test.
**Supplementary FIG. S6. Effect of cardiotoxic LC on pharyngeal mitochondrial membrane potential.** Representative images illustrate the tetramethylrhodamine, methyl ester (TMRM) accumulation, which appeared brighter in pharyngeal healthy cells of vehicle fed worms (A), and was lost after the treatment with cardiotoxic LC (B), but not myeloma protein (C), as indication of depolarization of the mitochondrial membrane potential. A similar effect was observed with H$_2$O$_2$, used as positive control (D). Scale bar, 50 µm.
Supplementary FIG. S7. Individual mitochondrial from heart muscle tissue of cardiac AL patients (A-C) and of a patient with dilatative cardiomyopathy (D). Ultrastructural details from representative TEM images of individual mitochondria from insets of Figure 4. Mitochondria in the myocardium of AL patients show enlarged size and either disruption (A) or nearly total loss of cristae (B, C). In the myocardium of a patient affected with severe non-amyloid cardiomyopathy (D), mitochondria show well preserved morphology. Scale bar, 200 nm.
Supplementary FIG. S8. Protective effect of TETRA and NAC on the pharyngeal pumping dysfunction caused by cardiotoxic LC. Cardiotoxic LC (H7-BJ) and myeloma (MM2-BJ) in 10 mM PBS, pH 7.4, were administered to worms (100 worms/100 µL) at 100 µg/mL alone or with 50 µM TETRA or 5 mM NAC. Control worms received vehicle alone (Vehicle). After incubation for 2 h in the absence of OP50 E. coli, worms were plated on NGM plates seeded with bacteria. Pharyngeal pumping was evaluated 20 h after plating and expressed as pumps/minute. **p<0.01 vs. vehicle and °° p<0.01 vs. cardiotoxic LC, according to one-way ANOVA and Bonferroni’s post hoc test.
Supplementary FIG. S9. Colocalization of nuclear staining and DAF-16::GFP in transgenic TJ356 worms fed cardiotoxic LC. Representative images depict in (A) the blue fluorescence of nuclear staining and in (B) the green fluorescence provided by DAF-16::GFP labeling. The nuclear localization of DAF-16::GFP was demonstrated by the colocalization of the two stainings in panel (C). Scale bar= 100 µm.
Supplementary FIG. S10. Effect of hydrogen peroxide on DAF-16 nuclear translocation and HSP-16.2 and SOD-3 pharyngeal expression. Transgenic worms were fed with 1 mM H$_2$O$_2$ for 30 min. Worms were then plated on NGM agar plates seeded with *E. coli*. (A) Representative image of DAF-16::GFP distribution in transgenic TJ356 *C. elegans*. Scale bar= 50 µm. (B) HSP-16.2 and SOD-3 expression, as visualized by GFP-fluorescence, in transgenic CL2070 and CF1553 worms, respectively. Scale bar= 100 µm.