A Caenorhabditis elegans-based assay recognizes immunoglobulin light chains causing heart amyloidosis

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A *Caenorhabditis elegans*-based assay recognizes immunoglobulin light chains causing heart amyloidosis

Short title: Amyloid light chain cardiotoxicity

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Key Points

- *C. elegans* specifically recognizes as toxicants cardiotoxic LC.

- This is an innovative model for studying the heart-specific toxicity of amyloidogenic LC and developing new therapeutic strategies.

ABSTRACT

Poor prognosis and limited therapeutic options characterize immunoglobulin light chain (AL) amyloidosis with major heart involvement. Reliable experimental models are needed to study light chains (LC)-heart interactions and to explore strategies for prevention of cardiac damage. We have exploited the nematode *C. elegans* as a novel tool, since its pharynx is evolutionarily related to the vertebrate heart. Our data demonstrate that the pharyngeal pumping of *C. elegans* is significantly and selectively reduced by LC from AL patients suffering from cardiomyopathy, but not by amyloid LC with different organ tropism or non-amyloidogenic LC from multiple myeloma. This functional alteration is dependent on the LC concentration and results in persistent pharyngeal dysfunction and in a significant reduction of the worms' lifespan. These manifestations are paralleled by an increase of mitochondrial reactive oxygen species and can be prevented by treatment with antioxidant agents. In conclusion, these data indicate that this nematode-based assay is a promising surrogate model for investigating the heart-specific toxicity of amyloidogenic LC and for a rapid screening of new therapeutic strategies.

INTRODUCTION

Immunoglobulin light chain (AL) amyloidosis is the most frequent systemic form in Western countries [1,2] and is caused by aggregation and deposition of monoclonal immunoglobulin light chains (LC) produced by a bone marrow plasma cell clone and transported to target organs through the bloodstream. The clinical picture in this form is heterogeneous and depends on which organs are targeted by fibril deposition. Cardiac involvement is present in up to 75% of patients and is the main prognostic determinant, leading to death for chronic heart failure or fatal arrhythmias [3].

Chemotherapy, by reducing the circulating amyloidogenic LC through suppression of the plasma cell clone, leads to extended survival and improved organ function [3,4]. However, its benefits to patients with severe heart dysfunction are minimal, because they are too fragile to receive aggressive treatments and do not survive long enough to benefit from any responses to therapy[5].

Clinical and experimental observations suggest that in AL amyloidosis, the soluble precursor protein itself may exert direct cardiotoxicity that significantly contributes to organ dysfunction in addition to the damage caused by cardiac infiltration of amyloid fibrils[4-8]. Cellular functional alterations, oxidative stress[7-9] and activation of specific signal transduction pathways [8] were found as prominent aberrant features *in vitro*. Possibly, both host-related factors and intrinsic LC characteristics are required to cause organ toxicity. *In vitro* data suggest that LC associated with cardiomyopathy have an intrinsic and specific cardiotoxic potential. Although the use of certain LC germline genes has been associated with specific organ targeting [10], including the heart [11], no specific features related to LC cardiotoxicity have been described yet, and predicting a protein's ability to target the heart is currently impossible.

The development of experimental models able to reproduce LC cardiotoxicity remains a major and urgent need to facilitate early diagnosis and investigate the mechanisms of toxicity in order to develop novel therapeutic strategies.

We used *Caenorhabditis elegans* as *in vivo* model to investigate the effect of cardiotoxic LC and clarify the underlying mechanisms of toxicity. *C. elegans* was chosen because its pharynx is considered to be evolutionarily related to the vertebrate heart and its muscle cells have autonomous contractile activity, reminiscent of cardiac myocytes [12]. The rhythmic contraction and relaxation of the nematode's pharyngeal muscle, *pharyngeal pumping*, is responsible for the ingestion and transport of food from the mouth to the intestine [13]. 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 which induce the production of cellular stress proteins [14].

We evaluated the effect on pharyngeal pumping of soluble amyloidogenic LC with different organ tropisms from AL patients and non-amyloidogenic LC from multiple myeloma subjects. Only amyloidogenic LC that are cardiotoxic in patients caused a specific impairment of the pharyngeal pumping rate of *C. elegans*. These manifestations are paralleled by an increase of mitochondrial reactive oxygen species and can be prevented by treatment with antioxidant agents. The data indicate that the nematode-based assay is a promising model for investigating heart-specific toxicity of amyloidogenic LC and for a rapid screening of the potential drugs and novel therapeutic approaches.

METHODS

Patients' samples

Urine, serum and bone marrow plasma cells were obtained from patients during routine diagnostic procedures at the Amyloid Research and Treatment Center, Foundation IRCCS Policlinico San Matteo (Pavia, Italy). Acquisition, storage and use of biological samples were approved by the Institutional Review Board. Written informed consent was received from participants prior to inclusion in the study. The study was conducted in accordance with the Declaration of Helsinki. The presence of tissue amyloid deposits and amyloid organ involvement were defined according to the International Consensus Panel Criteria [15,16]. LC cardiotoxicity was evaluated on the basis of clinical, instrumental (echocardiography) and biochemical parameters [17]. The clinical characteristics of the patients included in the study are reported in Table 1.

Human monoclonal amyloidogenic cardiotoxic LC (H), amyloidogenic non-cardiotoxic LC and non-amyloidogenic LC from multiple myeloma patients without amyloidosis (MM) were isolated from 24 h urine collected from patients (Bence Jones, BJ) and/or from serum (s) or produced, as recombinant proteins (r), in a bacterial system (see Supplemental Methods). The homogeneity of all the purified LC was assessed by 12% SDS PAGE and Western blot. In addition, mass spectrometry analysis, physicochemical studies and the examination of the oligomerization state were performed on prototypic proteins, *i.e.* recombinant cardiotoxic (H3-r) and non-cardiotoxic (K3-r) amyloid proteins and on BJ proteins purified from urine of a patient suffering from AL cardiomyopathy (H6-BJ) and from a patient with multiple myeloma (MM2-BJ) (see Supplemental Methods)[18]. The biochemical characteristics of these proteins (*e.g.* size, molecular mass and calculated

isoelectric point) were reported in Supplementary Figure 1. All LC included in the study were λ isotype, which represent ~75% of amyloidogenic LC [19].

C. elegans experiments

Bristol N2 strain, from the *Caenorhabditis elegans* Genetic Center (CGC, University of Minnesota, USA) was propagated at 20°C on solid Nematode Growth Medium (NGM) seeded with *E. coli* OP50 (from CGC) for food [20]. To prepare age-synchronized animals, nematodes were transferred to fresh NGM plates on reaching maturity at 3 days of age and allowed to lay eggs overnight. Isolated hatchlings from the synchronized eggs (day 1) were cultured on fresh NGM plates at 20°C. For pumping rate assays, nematodes, L3-L4 larval stage, were collected with M9 buffer, centrifuged and washed twice with 5 mM PBS, pH 7.4, to eliminate bacteria. Incubation of the worms with LC was performed in the absence of *E. coli*, to avoid any potential interference between bacteria and the LC [14,21]. Worms were incubated with 1-200 µg/ml BJ or recombinant LC (100 worms/100 µl) in 10 mM PBS, pH 7.4, or with 50 µg/ml free LC from patients' serum, in 5 mM PBS, pH 7.4 (100 worms/100 µl). Control worms were incubated with 10 mM PBS, pH 7.4 (vehicle) only. The effect of eluate obtained from incubation of anti-free LC antibodies with serum of healthy donors was also considered. After 2 h, 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 from 2 h up to 48 h later. The pumping rate of worms treated with the eluate obtained from serum of healthy donors was not different from vehicle treated ones (data not shown). For this reason, 10 mM PBS, pH 7.4, was used as vehicle for all the experiments. To visualize the effect of LC on the pumping rate, the feeding assay was performed by monitoring the ability of worms to ingest multifluorescent beads (Supplemental Methods).

The effect of LC administration on pharyngeal cell viability was determined using the cell-impermeable dye propidium iodide, which can be fed to *C. elegans* (Supplemental Methods).

In selected experiments, worms were fed for 2 h with 100 µg/ml of H6-BJ alone or with the prototypic antioxidants N-acetyl-cysteine (NAC, 0.1-20 mM, Sigma-Aldrich) and L-ascorbic acid (5-568 µM, Sigma-Aldrich) in 5 mM PBS, pH 7.4. The effect of tetracycline hydrochloride (TETRA, 5-100 µM, Sigma-Aldrich) and epigallocathechin gallate (EGCG, 0.1-1000 µM, kindly provided by Indena, Italy), in 5 mM PBS, pH 7.4, both known to possess antioxidant properties, was also considered. Higher doses of drugs proved toxic for nematodes. The effect of 0.1 mM H_2O_2 for 30 min was also investigated. 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 conditions.

For lifespan experiments, L3 larval stage worms were fed for 2 h with: a) 100 µg/ml of MM2-BJ or 100 µg/ml H6-BJ; b)100 µg/ml H6-BJ plus the optimal dose of an antioxidant compound (5 mM NAC, 284 µM ascorbic acid, 50 µM TETRA or 100 µM EGCG); c) the optimal dose of antioxidant compound alone. Then nematodes were transferred onto fresh NGM plates seeded with *E. coli* in the presence of the same drug concentration. Control worms were exposed, under the same conditions, to vehicle alone. After 20 h nematodes were transferred to fresh NGM plates seeded with bacteria and the number of live worms was scored (considered as day 0). To avoid overlapping generations, the worms were then transferred every day**,** in the absence of fluorodeoxyuridine, on NGM plates seeded with *E. coli* in the presence or absence of 5 mM NAC, 284 µM ascorbic acid, 50 µM TETRA or

100 µM EGCG, until they stopped laying eggs. The number of live worms was determined for each consecutive day until all worms were dead [22].

Mitochondrial production of reactive oxygen species (ROS)

The effect of LC on mitochondrial oxidant burden was evaluated by feeding worms with MitoSOX Red (Molecular Probes, Italy) [23,24]. Worms, L3-L4 larval stage, were incubated for 2 h with 100 µg/ml of MM2-BJ, H6-BJ, H3-r or K3-r, as described above. Negative control worms were fed vehicle alone whereas positive controls were fed 0.1 mM H_2O_2 to induce oxidant stress. To investigate the effect of antioxidant on mitochondrial ROS production, worms were incubated for 2 h with 100 µg/ml H6-BJ or for 30 min with 0.1 mM H_2O_2, in the absence or presence of 5 mM NAC, 284 µM L-ascorbic acid, 50 µM TETRA or 100 µM EGCG. The effect of drug alone was also determined. Nematodes were then transferred to NGM plates seeded with fresh bacteria as food and 10 µM MitoSOX Red. After 20 h, nematodes were transferred to fresh NGM plates seeded with OP50 and left for 1 h, so that residual dye could be washed out from the pharynx lumen. Nematodes were paralyzed with 1 mM levamisole, transferred to tubes containing 1 ml of M9 plus 1 mM levamisole, centrifuged and fixed in 4% paraformaldehyde in 5 mM PBS, pH 7.4, for 24 h at 4°C. Worms were then mounted on slides for microscopy and were observed by epifluorescence using an inverted fluorescent microscope (IX-71 Olympus) equipped with a CCD camera.

Statistical analysis

The data were analyzed using GraphPad Prism 4.0 software (CA, USA) by an independent Student's t-test and one-way ANOVA and Bonferroni's post-test analysis.

Vehicle and drug effects were compared using an independent Student's t-test, and the IC_{50} was determined using Prism version 4.0 for Windows (GraphPad Software, CA, USA). A p value < 0.05 was considered statistically significant.

Additional details are provided as **Supplemental Information**.

RESULTS

Cardiotoxic LC specifically impair pharyngeal pumping in C. elegans.

We first monitored the time-dependent effect of LC administration to *C. elegans* by using two representative BJ proteins: an amyloidogenic cardiotoxic protein (H6-BJ) and a non-amyloidogenic one (MM2-BJ), from a patient with multiple myeloma (Table 1 and Supplementary Figures 1-5). These proteins derive from two unrelated germline genes (*IGLV6-47* and *IGLV3-19*) and share only 74% amino acid identity. Their size, molecular mass and isoelectric point are shown in Supplementary Figure 1. H6-BJ and MM2-BJ were present in solution as small monomers or dimers, or at most trimers, and fibrils or higher aggregates were not present (Supplementary Figures 3 and 4).

Proteins were administered to worms for 2 h at 100 µg/ml, which is the representative concentration of circulating levels of free LC in AL patients, and were then plated on NGM plates seeded with OP50 *E. coli*. These experimental conditions did not affect the physiological pharyngeal contraction of worms[14]; in fact, the pumping rate of *C. elegans* incubated with vehicle, without bacteria (235.0 ± 3.6 pumps/min, N=30), as determined 2 h after plating, was not different from untreated ones which fed OP50 seeded on the plate for the same time (225.4±5.1 pumps/min, N=30).

The pharyngeal pumping of LC fed nematodes was scored at different times after plating worms on NGM plates seeded with bacteria. After 2 h, the pumping rate of H6-BJ-fed worms was already significantly reduced compared to vehicle-fed ones and a comparable inhibition was observed for up to 48 h (Figure 1A). This reduction in pumping motion was not accompanied by a modification of the worms' viability. Feeding worms with the non-amyloidogenic MM2-BJ did not cause reduction in the pumping rate nor affected the viability for every interval considered (Figure 1A). Therefore, cardiotoxic LC caused a

significant and prolonged reduction in pharyngeal contraction and a time-point of 20 h after plating was selected.

The inhibition to the pumping rate caused by H6-BJ was dose-dependent in the range of concentrations from 2.5 to 200 µg/ml, whereas MM2-BJ was not effective (Figure 1B). The effect of H6-BJ was significant from 10 µg/ml and reached the maximum at 100 µg/ml (Figure 1B). The pumping impairment caused by this protein concentration was visualized by evaluating the ability of worms to ingest fluorescent beads (Figure 1C and Supplemental Methods). In the pharynx of H6-BJ fed worms a strong reduction of the steady-state fluorescent signal was observed, compared to vehicle-fed animals (Figure 1C) indicative of a pumping impairment.

To further exclude the chance that the different toxicity observed in the above described experiments could be attributed only to extensive differences in the LC primary sequences, we also compared the effects of two recombinant proteins derived from patients with severe amyloid cardiomyopathy (H3-r) or overt nephrotic syndrome and renal insufficiency (K3-r) (Table 1). These two selected LC derived from the rearrangement of the same *IGLV1-44* germline gene, associated with a 5-fold increase in the odds of dominant heart involvement in AL amyloidosis[11] and have ~94% amino acid identity. Although both proteins proved able to inhibit the pumping rate, H3-r was more effective than K3-r at concentrations above 10 µg/ml (Figure 1D).

Notably, urinary and recombinant cardiotoxic LC had very similar effects on *C. elegans* pharynx, as indicated by the shape of the dose-response curves and IC_{50} values obtained (3.9± 1.2 µg/ml and 5.6 ± 2.1 µg/ml, for H6-BJ and H3-r, respectively, p= 0.294, Student's t- test) (Figure 1E).

Then, we evaluated the effect of LC with different organ tropism obtained from 15 AL and 5 MM patients (Table 1). Overall, 23 proteins were tested (8 BJ, 11 serum free LC, 4 recombinant free LC) (Table 1 and Figure 2). BJ proteins were tested at 100 µg/ml: LC that are cardiotoxic to patients significantly impaired the pumping rate of nematodes (191.0±3.5 and 225.0±3.0 pumps/min for cardiotoxic LC and vehicle fed worms, respectively, $p<0.001$, one-way ANOVA), whereas no effect was observed with LC from subjects affected by amyloidosis involving organs other than the heart (*e.g.* kidney) (226.5±1.5 pumps/min, $p<0.01$ *vs.* cardiotoxic LC, one-way ANOVA) or with multiple myeloma (228.2±4.0 pumps/min, $p<0.01$ *vs.* cardiotoxic LC, one-way ANOVA) (Figure 2A). The impairment of pharyngeal pumping caused by LC purified from patients' serum was then explored. Despite the lower dosage used (50 µg/ml), due to reduced recovery of LC from serum[25], the specific effect of cardiotoxic LC was also observed in this experimental setting (190.1.0±2.0 and 207.2±2.0 pumps/min for cardiotoxic LC and vehicle fed worms, respectively, $p<0.001$, one-way ANOVA) (Figure 2B). Similarly, cardiotoxic LC (100 µg/ml), produced as recombinant proteins in a bacterial system, caused a significant pumping rate reduction (186.7±1.5 and 225.3±23.0 pumps/min for cardiotoxic LC and vehicle fed worms, respectively, $p<0.001$, one-way ANOVA) (Figure 2C). Overall, these data indicate that cardiotoxic LC obtained from different patients specifically trigger a pharyngeal dysfunction in *C. elegans* independent of their origin: serum, urine or recombinant.

To determine whether the impairment of pumping function caused by cardiotoxic LC may be related to a reduction of viable cells in the pharynx, worms were fed propidium iodide, a fluorescent dye that enters and stains dead cells (Supplemental Materials). In the pharynx region of H6-BJ fed worms, but not in the control ones, we observed a red fluorescence-positive signal due to staining of dead cells (Supplementary Figure 6D). This cell death

level was comparable to that observed by exposing worms for 30 min to 10 mM H_2O_2 (Supplementary Figure 6F) which caused a significant impairment of the pharyngeal pumping rate (226.7 ±2.0 and 190.1 ±1.0 pumps/min for vehicle and H_2O_2 fed worms respectively; $p<0.01$ one-way ANOVA, N=30 worms/group).

Recent data indicated that soluble LC caused a marked reduction in the lifespan of zebrafish [26,27]. We then analyzed whether the pharyngeal dysfunction induced by cardiotoxic LC could affect, in time, nematode survival. The administration of H6-BJ, but not MM2-BJ, significantly reduced the worm's survival (median survival: 14 days for vehicle; 9 days for H6-BJ, p=0.024 *vs*. vehicle, Log-rank test; 14 days for MM2-BJ, p=0.072 *vs.* H6-BJ and p=0.53 *vs*. vehicle, Log-rank test) (Figure 3A).

Functional damage caused by cardiotoxic LC is related to their ability to generate oxygen radicals. We investigated whether the ability of cardiotoxic LC to reduce the pharyngeal muscular pumping of the nematodes was related to their propensity to form specific, partially folded intermediates, *i.e.* small, soluble oligomeric assemblies. The physicochemical properties and the oligomerization state of the above described recombinant and BJ proteins were considered (see Supplemental Methods). The electrophoretic data, Western blot and mass spectrometry analysis indicated that these LC preparations were highly homogeneous, revealing a molecular mass in agreement with the theoretical mass (Supplementary Figure 1). Cardiotoxic and non-cardiotoxic LC showed a comparable degree of stability and a similar pattern of secondary structures (Supplementary Figure 2). The size and oligomerization state of recombinant and urinary LC were evaluated by size-exclusion chromatography and dynamic light scattering studies and indicated that similar monomeric and dimeric species were present in both, cardiotoxic

and non-cardiotoxic LC solutions (Supplementary Figures 3-4). In addition, LC with different organ tropism, had a similar exposure of hydrophobic regions (Supplementary Figure 5). These data indicated that the ability of cardiotoxic LC to specifically impair the nematode's pharynx contraction was not related to a peculiar protein stability, assembly as well as to differences in hydrophobic regions exposure.

We then explored whether LC toxicity can be related to their propensity to produce ROS [7,8,28] thus triggering an oxidative damage. Oxygen free radicals produced by MM2-BJ, H6-BJ, H3-r and K3-r were detected by electron paramagnetic resonance (Supplemental Methods and Supplementary Figure 7) and the spectra obtained indicated that cardiotoxic LC exhibited a markedly different behaviour compared to non-cardiotoxic ones. In particular, H6-BJ produced a greater amount of OH and 2OH radical species than MM2-BJ (Supplementary Figure 7). Notably, whereas the tiny amount of radicals produced by non-cardiotoxic LC quickly disappeared, cardiotoxic proteins continuously generate oxygen radicals (Supplementary Figure 7), which are known stressors, causing the pharyngeal pumping inhibition in *C. elegans*.

To further explore the oxidative stress pathogenic hypothesis, we next investigated mitochondria, to clarify whether the stressful condition generated by cardiotoxic LC in *C. elegans'* pharynx can result in ROS generation within its sub-cellular compartments. In fact, an excessive production of ROS within mitochondria can induce oxidative damage that may be related to the pharyngeal dysfunction [29]. The pharyngeal pumping dysfunction caused by the cardiotoxic H6-BJ and H3-r proteins was accompanied by a significant increase in the fluorescence of MitoSOX, a mitochondria-specific redox-sensitive dye, indicative of an enhanced oxidant burden (Figure 3B and Supplementary Figure 8). This oxidant level was comparable to that generated by exposing worms to 0.1 mM H_2O_2

(Figure 3B) which caused a significant reduction in the pharyngeal pumping rate (228.3 ±2.0 and 196.1 ±1.2 pumps/min for vehicle and H_2O_2 fed worms respectively; $p<0.01$ one-way ANOVA, N=30 worms/group). No specific MitoSOX fluorescence was observed in the pharynx of worms fed the MM2-BJ protein or vehicle (Figure 3B). Thus, the pharyngeal pumping dysfunction induced by cardiotoxic LC is associated with enhanced mitochondrial ROS production.

These observations prompted us to explore an alternative approach to abolishing or reducing LC toxicity. The effect of the prototypic antioxidants N-acetyl-cysteine (NAC) and ascorbic acid was investigated. We also examined the polyphenolic green tea constituent epigallocathechin gallate (EGCG) and tetracycline hydrochloride (TETRA), both known to possess antioxidant properties [30,31]. All these compounds reduced the inhibition of pharyngeal pumping caused by H6-BJ in a dose-dependent manner, although with different potency (Figure 4 A-D). The highest IC_{50} values were measured for compounds displaying only antioxidant effects (3 ± 1.1 mM and 150 ± 4.5 µM for NAC and ascorbic acid, respectively), whereas EGCG and TETRA had much lower IC_{50} (11 ±1.2 µM and 18 ±1.1 µM, for EGCG and TETRA, respectively) (Figure 4). Five mM NAC, 284 µM ascorbic acid or 100 µM EGCG completely abolished the pharyngeal impairment caused by the H6-BJ, whereas 50 µM TETRA significantly counteracted this inhibition but did not reverse it (Figure 4E). The ability of all these drugs to neutralize the pumping dysfunction caused by cardiotoxic LC was accompanied by a strong reduction of ROS generation into pharyngeal mitochondria, as indicated by the inhibition of MitoSOX-related fluorescence (Supplementary Fig. 9). Drugs alone did not affect the pumping rate (Figure 4E), nor the increase of pharyngeal mitochondrial oxygen burden (Supplementary Figure 9). We also investigated whether ROS scavengers prolonged the lifespan of nematodes fed

cardiotoxic LC. Daily repeated administration of 5 mM NAC or 284 µM ascorbic acid significantly prolonged the survival of H6-BJ-treated worms, completely restoring their natural lifespan (Supplementary Figure 10), indicating that the prevention of ROS generation by cardiotoxic LC can modify the overall toxicity of the proteins. However, these protective effects were not observed with repeated administration of 50 µM TETRA or 100 µM EGCG, even at lower doses, because they resulted toxic to worms. These findings are in agreement with the reported anti-helminthic activity of chronic administration of TETRA and EGCG [32,33].

DISCUSSION

Although amyloid deposition is a defining feature of cardiac amyloidosis, the presence of fibrils is not sufficient to explain the cardiotoxicity observed in AL patients [4,5]. Clinically, the rapid improvement or worsening of cardiac function, which parallels variations in serum LC concentration in the absence of changes in amyloid load, cannot be explained without attributing a toxic role to pre-fibrillar, soluble LC species [4,5]. The extreme heterogeneity of the clinical presentation, together with the complex interplay of biological, biochemical and biophysical factors in determining the disease, makes it highly desirable to develop controllable model organisms for the investigation of the pathogenic mechanisms underlying AL *in vivo.* A transgenic mouse has recently been generated [34], however, it does not adequately reproduce AL amyloid cardiomyopathy, thus precluding its use for an efficient study of the mechanisms of heart damage. The toxicity exerted by soluble LC has been confirmed in *in vitro* [6-9] and *in vivo* [26,27,34] experiments. Recent data obtained in zebrafish indicated that soluble LC species caused cardiomyopathy as well as a marked dose-dependent reduction in lifespan [26,27].

In order to study the pathogenic role of exogenous, soluble LC in AL cardiomyopathy, we here describe the use of *C. elegans* as a novel animal model. The limitation of this model is its distance from vertebrates, and translation to human pathology requires caution. However, many human stress pathways are replicated in worm, rendering this animal a rapid and versatile system for exploring the mechanisms underlying various complex diseases [35]. The suitability of this model was supported by the knowledge that the worm's pharynx possesses an automatic contractile activity, reminiscent of the vertebrate heart, of which it is considered an ortholog [12,13]. We demonstrated that soluble LC from patients suffering from amyloid cardiomyopathy, but not non-cardiotoxic LC, are specifically

recognized as toxicants by the worm's pharynx. The effect of cardiotoxic LC was dose-dependent and became maximal at concentrations in the range of those commonly found in blood of patients with AL amyloidosis [4,5]. Interestingly, damage was already exerted by low concentrations of toxic LC.

The inhibition of the worm's pharyngeal pumping persisted up to 48 h after exposure and was accompanied by cell death suggesting a profound subversion of the pharynx cells' function. In time, this resulted in a significant reduction in the worms' lifespan.

Interestingly, we observed that toxic LC did not display a peculiar protein folding, nor propensity to form aggregates of a different size compared to the non-toxic LC species. The lack of differences in oligomerization, secondary structure, stability and surface hydrophobicity between cardiotoxic and non-cardiotoxic LC lead us to hypothesize that toxicity may proceed through common super-secondary structures linked to the proteins' primary sequence. Indeed, the nematode assay proved to be very sensitive and specific in this aspect, being capable of discerning the few amino acid differences of two recombinant LC derived from the same germline gene (*IGVL1-44*), but with different organ tropism and toxicity (Figure 1D, K3r, from a patient with kidney involvement, and H3r, from a patient with cardiac dysfunction). This argument is in line with the notion that the LC sequence is a major determinant of specific organ targeting and damage[10,11] .

It is noteworthy that *C. elegans* has previously been employed as a model to study various amyloidoses [22,36-39]. In particular, our group has demonstrated that $A\beta_{1-42}$ oligomeric intermediates, but not monomers or fibrils, caused a specific impairment of the worm's pharyngeal function [21,39]. The common pattern of damage exerted by cardiotoxic LC and Aβ oligomers on the worm's pharyngeal cells supports the hypothesis of shared pathways of toxicity between the two proteins. One of these, as described later, may involve

mitochondria, which are known early targets of damage in Alzheimer's disease [40,41], and are particularly abundant in both brain and cardiac muscular cells.

In the zebrafish AL model, the cardiotoxic effect was paralleled by the activation of the oxidative stress pathway [26], confirming the data obtained from other *in vitro* studies [6-8]. Also in our model, dysfunction caused by cardiotoxic LC was associated with oxidant stress; this, in turn, was related to an increase in the mitochondrial oxidant burden in the pharynx. This organ, similar to the heart, is a mitochondrial-rich tissue, due to mitochondrial crucial role as continuously supplier of energy during the contraction [12,23]. Mitochondria are highly sensitive to oxidative damage, and the stressful conditions produced by cardiotoxic LC in the pharynx, induced by ROS formation, can result in dramatic reduction of ATP availability for cardiomyocyte contraction with loss of organ function. The causal role of oxidative damage in dysfunction is supported by the fact that antioxidant drugs, which counteract ROS-induced damage, prevent the pharyngeal functional impairment caused by cardiotoxic LC, thus reducing the burden of mitochondrial oxygen radicals. All tested compounds are effective in protecting worms from LC toxicity. From a translational point of view, the data obtained with EGCG and TETRA are of particular interest. These drugs were reported to influence the toxic properties of several amyloidogenic proteins [31,42-47]. TETRA, beside their ability to disrupt and/or inhibit amyloid fibril formation, also hinder the oxidative stress, by inhibiting ROS generation and stimulating endogenous antioxidant enzymes, such as superoxide dismutase and glutathione peroxidase[31]. Preliminary data indicate that EGCG can produce clinical benefits with possible reduction of the cardiac amyloid deposits in patients with AL amyloidosis [48,49]. The efficacy of EGCG and TETRA is currently being evaluated in AL patients in Phase II Clinical Trials (ClinicalTrials.gov Identifier: NCT01511263 and

NCT01677286, respectively) [48,49]. Our data indicate that EGCG and TETRA exert a protective effect against LC toxicity. However, at variance with NAC and ascorbic acid, when administered chronically they did not restore the natural lifespan of *C. elegans*. This is essentially due to the reported peculiar specie-specific toxic effect of both EGCG and TETRA[32,33].

Overall, our work shows that *C. elegans* can be considered a "biosensor", suitable for a rapid and inexpensive assessment of the cardiotoxic potential of LC. Besides allowing the study of the molecular and functional consequences of exposure to LC, we anticipate its possible use for screening LC cardiotoxicity in basic research and translational applications. As an example, given its sensitivity to small sequence variations (Figure 1D), it may disclose the functional consequences of manipulation of the LC's primary sequence (directed mutagenesis) or the introduction of post-translational modifications. This would be of paramount importance for the precise definition of the toxic determinants in LC. The pharmacological data show that our assay is also a convenient way to screen the effect of different drugs and to elucidate their molecular mechanisms of action.

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AUTHORSHIP

Contributions: L.D., P.R., F.L., E.dF., L.C., E.G. designed and carried out experiments, analyzed results and wrote the manuscript. M.R., F.F., A.C. and A.dF. carried out experiments. V.P., G.P. and V.V. helped design experiments and reviewed the manuscript. G.M. and M.S. designed experiments, interpreted results and wrote the manuscript.

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Table 1. Clinical and biochemical characteristics of patients at diagnosis of immunoglobulin light chain amyloidosis (AL) or multiple myeloma (MM).

					Protein Source							Cardiac Parameters				
Code	Gender, age	Cardiac stage°	Diagnosis	Organs Involved°°	Recombinant	BJ	Serum	Serum λ FLC (mg/l)	κ/λ FLC ratio	Proteinuria (g/24h)	Creatinine (mg/dl)	NT-proBNP (ng/l)BNP* (ng/L)	cTnI (ng/ml)	IVS (mm)	PW (mm)	EF (%)
H-1	M, 54	III	AL	H, PNS, ST		x	x	839	0.0018	0.98	0.74	1444	0.222	16.8	16	70
H-2	F, 72	III	AL	H			x	769	0.02	0.23	1.58	31441	0.9	16	16	55
H-3	M, 65	III	AL	H	x			252	0.09	0.18	1.76	4491	0.35	16	16	42
H-4	M, 46	II	AL	H	x			185	0.08	0.35	1.50	4942	0.08	18	18	40
H-5	M, 72	III	AL	H, K			x	383	0.05	8.10	2.73	21587	1.18	14.9	14.4	61
H-6	M, 74	III	AL	H		x	x	683	0.009	0.45	0.73	4300	0.2	15	15	42
H-7	M, 45	III	AL	H		x	x	477	0.01	0.33	0.98	8882	0.16	19	19	45
H-8	F, 55	III	AL	H	x			388	0.005	0.14	0.83	5557	0.2	13	14	45
K-1	M, 69	I	AL	K			x	161	0.06	5.17	0.95	106	0.02	13.3	13.3	63
K-2	F, 60	I	AL	K			x	104	0.06	4.76	0.58	57.8	0.001	9.6	10.4	64
K-3	M, 68	I	AL	K	x			463	0.08	8.45	2.23	274	0.021	11	11	55
K-4	M, 72	I	AL	K			x	320	0.03	6.76	0.90	230	0.022	11.5	10	60
K-5	F, 63	I	AL	K			x	509	0.03	2.67	0.57	40.5	0.007	9.7	9.2	61
K-6	F, 69	I	AL	K		X		228	0.08	2.33	1.39	419.5	0.005	9	10	60
ST-1	F, 55	I	AL	ST		X		1220	0.0002	0.22	0.55	271	0.025	11.6	11.6	57
MM-1	F, 73		MM	-			x	618	0.003	0.13§	1.20	191	0.004	10.2	10.2	55
MM-2	F, 71		MM	-		X		6130	0.001	0.52§	2.07	42*	0.007	9	9	65
MM-3	M, 48		MM	-		X		573	0.011	1.87§	0.84	14.5	0.003	10	10.5	67
MM-4	M, 65		MM	-		X		1140	0.001	0.12§	0.89	201	n.a.	11	11	65
MM-5	M, 37		MM	-			x	500	0.01	0.11§	0.88	55	0	10	10	64

M, male; F, female; °According to Gertz et al. [15,16]; H, Heart; K, Kidney; ST, Soft Tissues; PNS, Peripheral Nervous System; BJ, Bence Jones; FLC, Free Light Chains; BNP, Brain Natriuretic Peptide; cTnI, cardiac Troponin I; IVS, Interventricular Septum; PW, Posterior Wall; EF, Ejection Fraction; n.a., not available. °°According to the International Consensus Panel criteria [15,16]. § Entirely constituted by BJP. Reference ranges: serum γ FLC <26.3 mg/l, κ/γratio 0.26-1.65; serum creatinine <1.18 mg/dl in men, <1.02 mg/dl in women; NT-proBNP [5] <332 ng/l; BNP, <50 ng/l; cTnI <0.04 ng/ml.

FIGURE LEGENDS

Figure 1. **Characterization of the effects of selected LC on the pumping rate.**
(**A**) Time-dependent effect of the amyloidogenic cardiotoxic protein (H6-BJ) and the non-amyloidogenic one (MM2-BJ) on the pumping rate of worms. Proteins, in 10 mM PBS, pH 7.4, were administered to worms at 100 µg/ml. Control worms received vehicle alone (Vehicle). Nematodes (100 worms/100 µl) were incubated with LC for 2 h in the absence of OP50 *E. coli*, and then plated on NGM plates seeded with bacteria. The pharyngeal pumping was scored at different times after plating (2-48 h). Data are expressed as the mean ± SE (N=20 worms/group). *p<0.01 *vs.* vehicle and MM2-BJ, Student's t-test. (**B**) Dose-response effect of 1-200 µg/ml of H6-BJ and MM2-BJ. Mean ± SE (N=40 worms/group). *p<0.01 *vs.* MM2-BJ, Student's t-test. (**C**) Effect of H6-BJ on the feeding behavior. Feeding assay was performed by monitoring the ability of worms to ingest multifluorescent beads. H6-BJ protein (100 µg/ml) in 10 mM PBS, pH 7.4, or vehicle alone (Vehicle) were administered to worms. Representative images, obtained from the overlay of a contrast phase and epifluorescence, indicated the presence of fluorescent beads (black arrows) in the pharynx of control worms but not in those fed H6-BK protein. (**D**) Dose-response effect of 1-200 µg/ml of recombinant cardiotoxic (H3-r) or non-cardiotoxic (K3-r) proteins. Mean ± SE (N=30 worms/group). *p<0.01 *vs.* K3-r, Student's t-test. (**E**) Comparison of the dose-response curves obtained for H3-r and H6-BJ proteins. IC_{50} values ± SD were reported. The two proteins, at 100 µg/ml, similarly inhibited the pumping rate of worms (from 235.0 ±3.6 pumps/min of vehicle to 177.6 ±3.2 pumps/min and 170.4 ±2.8 pumps/min for H6-BJ and H3-r, respectively).

Figure 2. Effect on the pumping rate of LC with different organ tropism, purified from different patients' urine and serum or obtained as recombinant.
Effect of (**A**) Bence Jones LC (100 µg/ml) purified from 8 patients (3 heart AL, 2 kidney AL, 3 MM), (**B**) serum free LC (50 µg/ml) purified from 11 patients (5 heart AL, 4 kidney AL, 2 MM) and (**C**) recombinant LC (100 µg/ml) obtained from 4 patients (3 heart AL and 1 kidney AL) on pharyngeal pumping. Nematodes (100 worms/100 µl) were incubated for 2 h, in the absence of OP50 *E. coli,* with different amyloidogenic non-cardiotoxic or cardiotoxic LC or non-amyloidogenic LC from patients with multiple myeloma The pharyngeal pumping rate was scored 20 h after plating the worms on NGM agar plates seeded with fresh OP50 *E. coli* as food. Control worms received vehicle alone (Vehicle).

Each dot on the scatter plot represents the mean value of pumps/min obtained for each single protein from three independent assays (N=30 worms/assay). These values were used to calculate the mean ± SE for LC with the similar organ tropism (horizontal line) and to perform a statistical comparison across the different groups. ** $p<0.001$ *vs*. vehicle, ° $p<0.05$ and °° $p<0.01$ Non-Cardiotoxic *vs*. Cardiotoxic LC, according to one-way ANOVA followed by Bonferroni's *post hoc* test.

Figure 3. **Cardiotoxic LC affects nematode survival and pharyngeal ROS generation.** (**A**) Kaplan-Meier survival curves of worms treated with Vehicle, 100 µg/ml of amyloidogenic BJ cardiotoxic protein (H6-BJ) or 100 µg/ml of non-amyloidogenic BJ protein (MM2-BJ). Survival is expressed as a percentage of the initial population (three independent experiments, N=30 worms/group). Median survival: 14 days for Vehicle, 9 days for H6-BJ (p=0.024 *vs*. vehicle, Log-rank test), 14 days for MM2-BJ (p=0.072 *vs.* worms treated with H6-BJ and p=0.53 *vs*. Vehicle, Log-rank test). (**B**) Representative images obtained from the overlay of a contrast phase and MitoSOX fluorescence. N2 worms, at L3-L4 larval stage, were fed 2 h with vehicle, 100 µg/ml of MM2-BJ or H6-BJ. Positive control worms were fed 0.1 mM hydrogen peroxide (H_2O_2) for 30 min. Nematodes were then transferred to NGM plates seeded with fresh OP50 *E. coli* and 10 µM MitoSOX Red dye. 40x magnification was used. Arrows indicate the radical superoxide generation in the mitochondria of pharyngeal bulb of *C. elegans* fed cardiotoxic H6-BJ protein or H_2O_2.

Figure 4. **Protective effect of antioxidants, tetracycline hydrochloride and epigallocathechin gallate on the pharyngeal dysfunction caused by cardiotoxic LC.** Dose-dependent effect of (**A**) N-acetyl-cysteine (NAC), (**B**) ascorbic acid, (**C**) tetracycline hydrochloride or (**D**) epigallocathechin gallate (EGCG). Worms were fed for 2 h with 100 µg/ml amyloidogenic cardiotoxic protein (H6-BJ), in the absence or presence of increasing concentrations of drugs. Control worms were fed with vehicle alone (dotted line). The pharyngeal pumping rate was scored 20 h after plating on NGM plates, as described in the Methods section. IC_{50} value was calculated for each compound. (**E**) The effect of 5 mM NAC, 284 µM ascorbic acid, 50 µM tetracycline or 100 µM EGCG was determined by incubating worms for 2 h with 100 µg/ml H6-BJ. The effect of the administration of each compound, alone, at the same concentration and H6-BJ alone was also evaluated. The pharyngeal pumping rate was scored 20 h after plating on NGM plates, as described in the

Methods section. Control worms were fed vehicle alone (Vehicle). Mean ± SE (N=30). **p<0.01 and *p<0.05 *vs*. vehicle, °°p<0.01 *vs.* H6-BJ alone according to one-way ANOVA followed by Bonferroni's *post hoc* test.

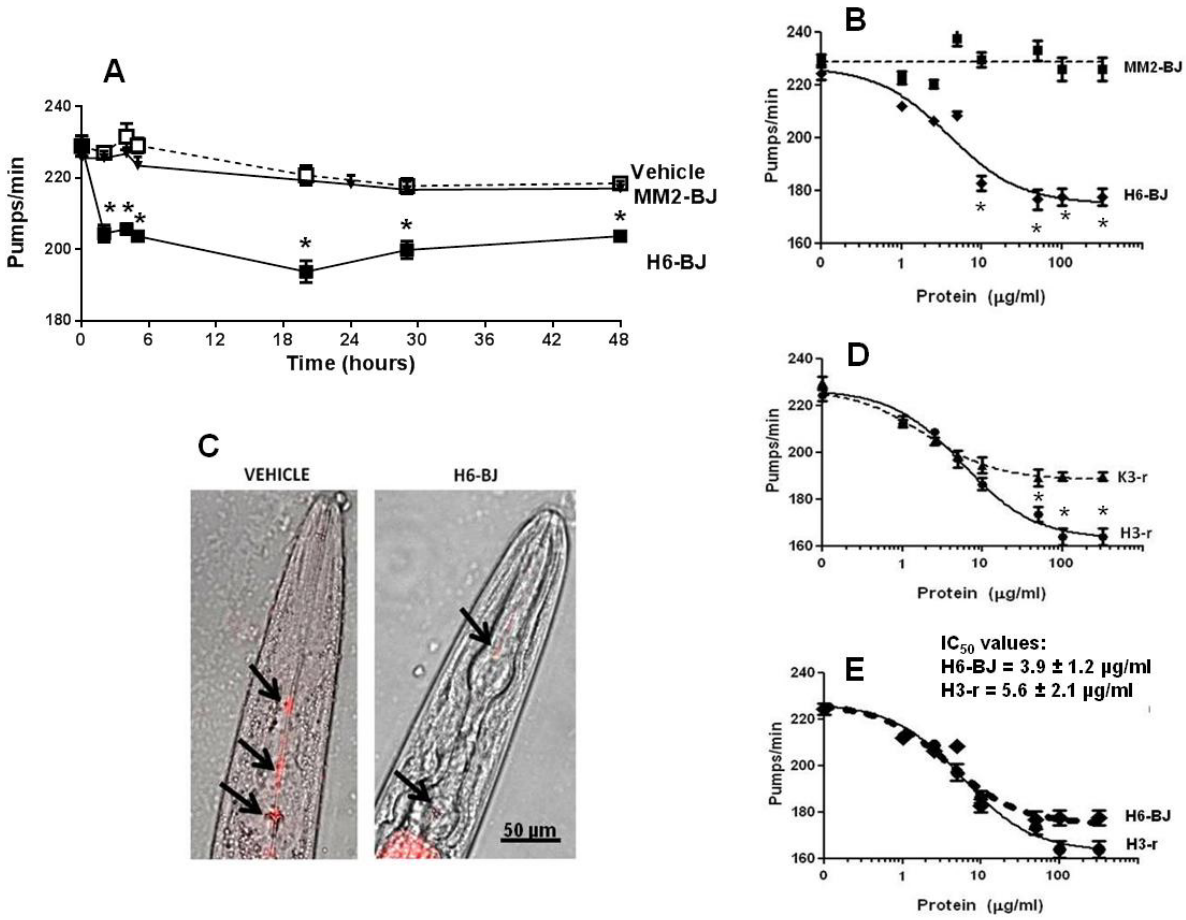

Figure 1

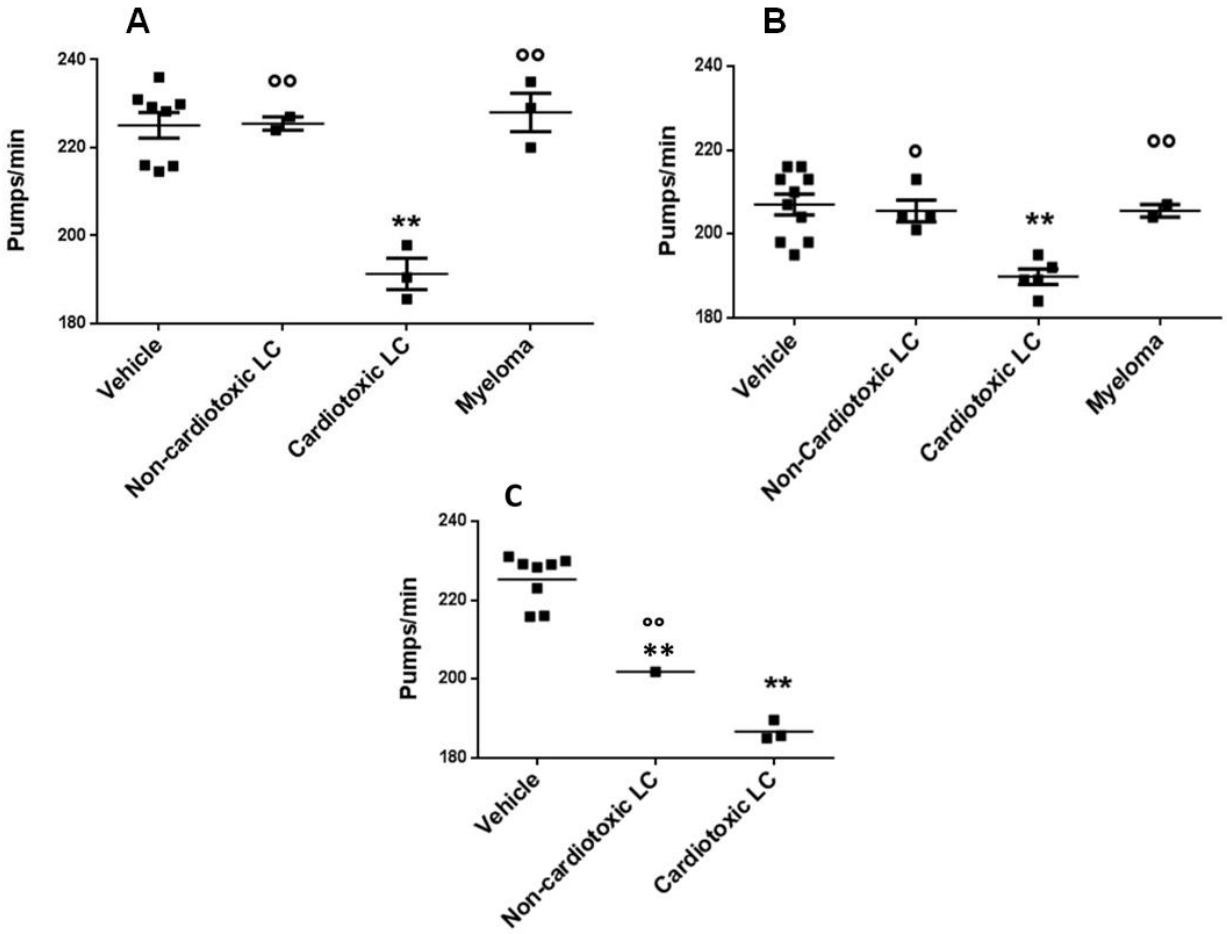

Figure 2

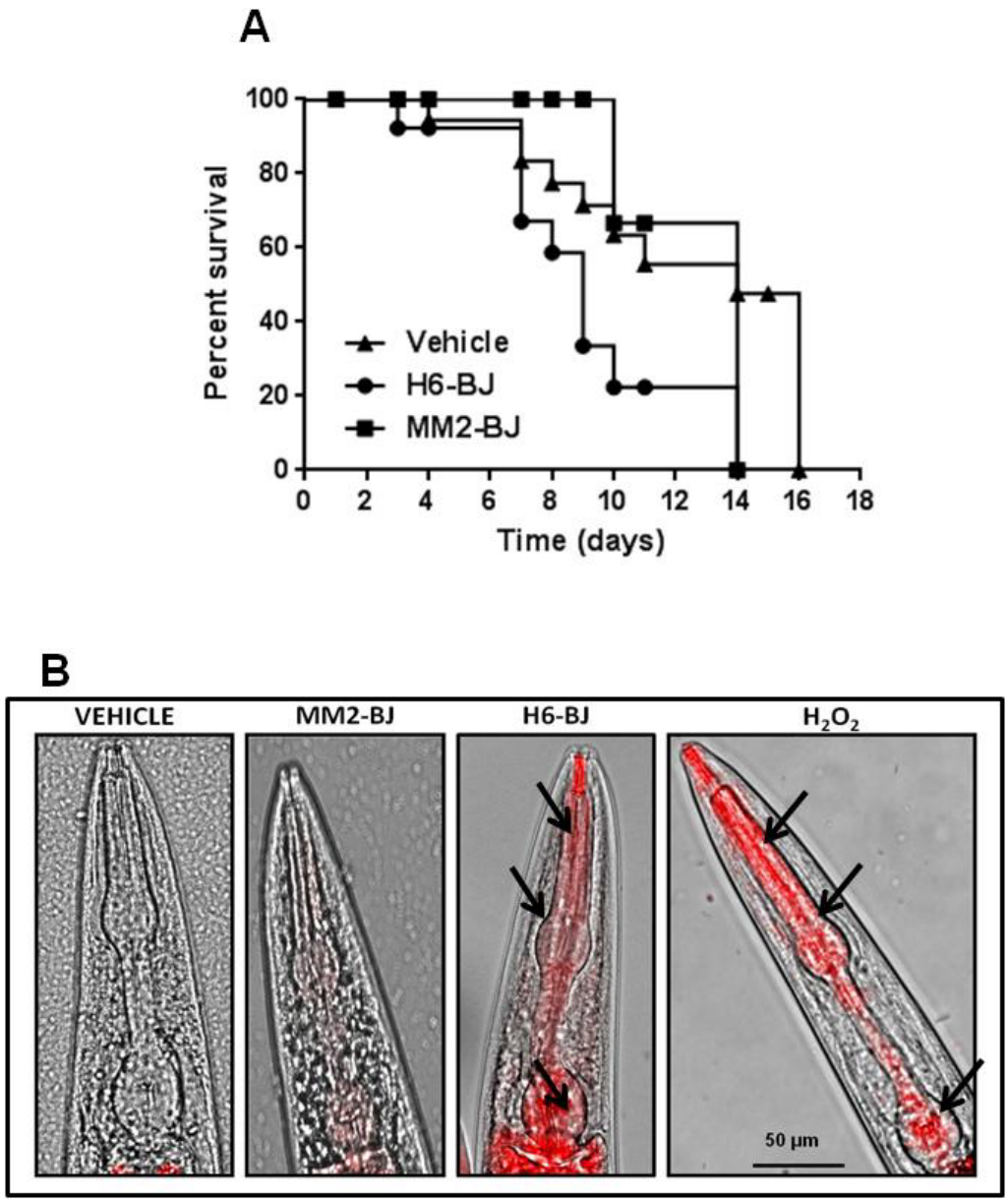

Figure 3

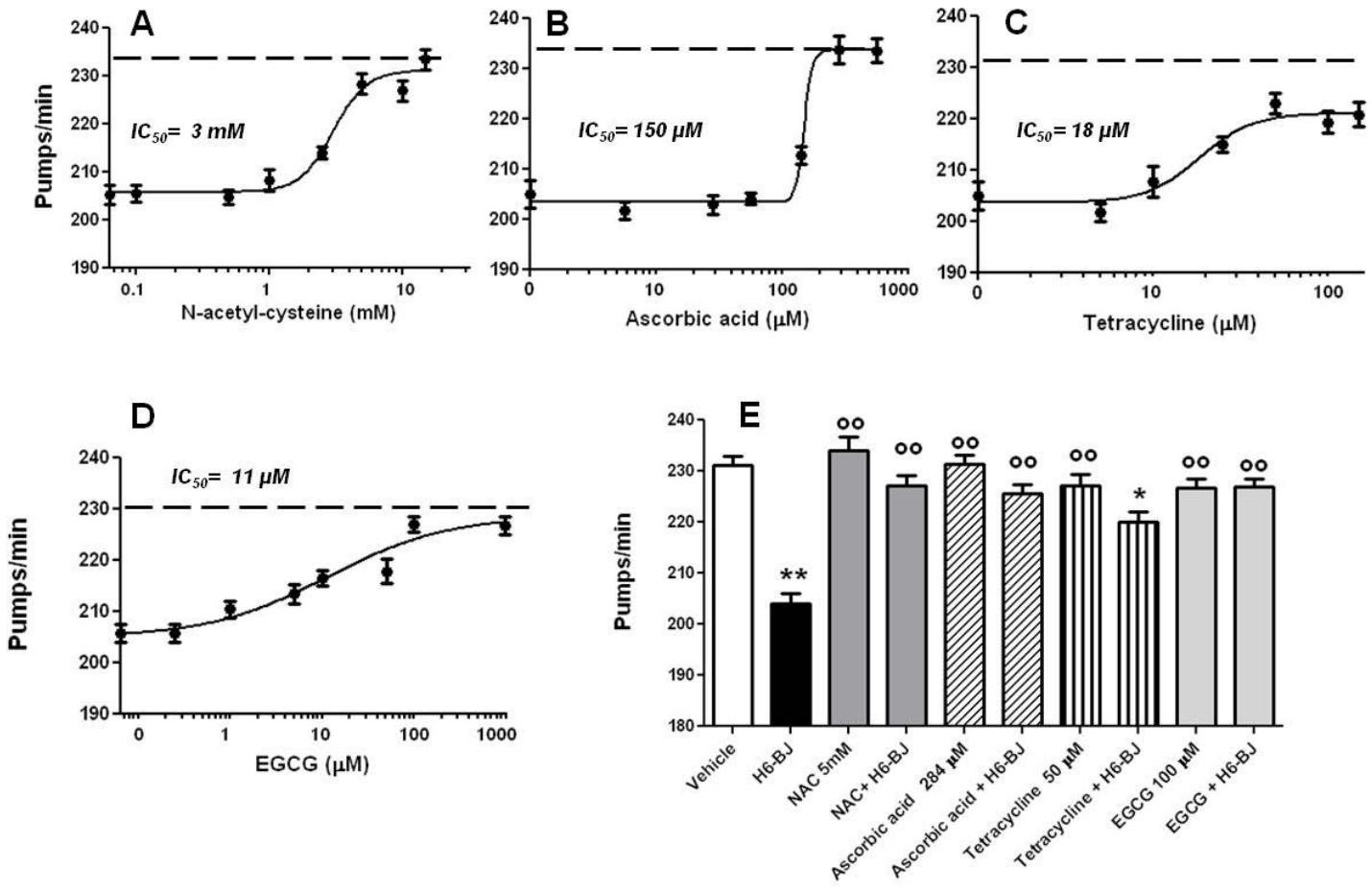

Figure 4