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Original article

Direct synthesis of 4-organylsulfenyl-7-chloro quinolines and their toxicological and pharmacological activities in *Caenorhabditis elegans*

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ABSTRACT

We describe herein our results on the synthesis and biological properties in *Caenorhabditis elegans* of a range of 4-organylsulfenyl-7-chloroquinolines. This class of compounds have been easily synthesized in high yields by direct reaction of 4,7-dichloroquinoline with organylthiols using DMSO as solvent at room temperature under air atmosphere and tolerates a range of substituents in the organylsulfenyl moiety. We have performed a toxicological and pharmacological screening of the synthesized 4-organylsulfenyl-7-chloroquinolines *in vivo* in *C. elegans* acutely exposed to these compounds, under *per se* and stress conditions. Hence, we determined the lethal dose 50% (LD_{50}), in order to choose a nonlethal concentration (10 μM) and verified that at that concentration some of the compounds depicted protective action against the induced damage inflicted by paraquat, a superoxide generator. Two compounds (**3c** and **3h**) reduced the toxicity inflicted by paraquat above survival, reproduction and longevity of the worms, at least in part, by reducing the reactive oxygen species (ROS) generated by the toxicant exposure. Besides, these compounds increased the quantities of superoxide dismutase (SOD-3::GFP) and catalase (CTL-1,2,3::GFP), antioxidant enzymes. We concluded that the protective effects of the compounds observed in this study might have been a hormetic response dependent of the transcriptional factor DAF-16/FOXO, causing a non-lethal oxidative stress that protects against the subsequently damage induced by paraquat.

1. Introduction

It is well known that reactive oxygen species (ROS) such as superoxide radical (O_2•−), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH•−) are capable of causing damage that increases gradually with aging in many organisms, originating alterations in a variety of molecules (lipid, protein, and DNA) from invertebrates to humans [1,2]. The broad spectrum of severe diseases in which an important role is played by reactive oxygen species, such as cancer, Alzheimer and atherosclerosis [3] leads to the necessity of searching for drugs that effectively neutralize/modulate ROS. Actually, 85% of the drugs available in the market come from synthetic origins and 62% of these are heterocyclic compounds. Among these, 91% contains nitrogen and 24% contains sulfur [4]. The synthesis of new compounds that can treat different ROS-associated pathologies is relevant and, to reach that, the insertion of different substituents into a chemical structure that has described pharmacological properties is a common synthesis practice. In this context, quinolines have been described as a very successful molecule in terms of pharmacological properties [5]. A number of compounds based on the quinoline ring have been known since the late 1800 s, such as quinidine (a class I antiarrhythmic agent), chloroquine, hidroxychloroquine, primaquine (antimalarial drugs) and quinine (antipyretic drug).

Similarly, organic compounds with chalcogen atoms in their structure, such as sulfur, are well known by their antioxidants properties. Many studies have demonstrated efficacious treatment with these type of compounds against disease models associated with oxidative stress [6–10]. Their ROS-scavenging activity is probably due to their high nucleophilicity. There are in the literature some methodologies for the synthesis of sulfur-containing quinolines, however these methodologies have some disadvantages,

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including long reaction times and harsh conditions [11–16,21]. Consequently, the search for new and efficient methodologies for the synthesis of novel sulfur-containing quinolines remains an important challenge for synthetic organic chemistry.

Some level of toxicity for these organochalcogens compounds are related in the literature [7,17–19] and, for that reason, toxicological screenings are needed in order the refine and reduce the number of animals involved in this type of research. The nematode *Caenorhabditis elegans* came as an alternative because of its easy and inexpensive maintenance in the laboratory, based on a diet of *Escherichia coli*. The entire life cycle, from an egg to an adult producing more eggs, takes just 3.5 days at 20 °C. Population growth is fastest at 20 °C, with brood sizes of ~300 produced over approximately a 4-day period [20]. Remarkably, the relatively straightforward generation of knockout strains for genes of interest and of transgenic worms expressing green fluorescent tagged proteins (GFP) are very useful for protein expression or localization studies. Remarkably, it was the first multicellular organism to have its genome completely sequenced, which has been found to have a high level of conservation with the vertebrate genome [20–22]. This includes the antioxidant system.

For instance, the *sod-3* gene, which encodes the mitochondrial superoxide dismutase, and *ctl-1 and ctl-2,* that encode the cytosolic and peroxisomal catalase, posses 97, 97 and 98% of homology with the human iso forms, respectively [23,24]. Such proteins can be used as markers to access the redox state of *C. elegans* [25–28]. The *sod* and *ctl* isoforms are, under oxidative stress response, regulated by the FOXO homolog DAF-16, a transcription factor widely related as essential for normal development, reproduction and lifespan in *C. elegans* [29].

We describe herein the direct synthesis of 4-organylsulfenyl-7-chloro quinolines by reaction of 4,7-dichloroquinoline with organylthiols using DMSO as solvent at room temperature under air atmosphere. Additionally we investigated the toxicological and pharmacological effects of the synthesized 4-organylsulfenyl-7-chloro quinolines in *C. elegans*. We hypothesized that our thioquinolines could act by indirectly neutralizing oxygen reactive species by a hormetic response induced by low ROS formation, which in turn upregulates SOD-3 and CTL-1,2,3 possibly via DAF-16 transcription factor, protecting the worms against the paraquat-induced oxidative damage in the survival, reproduction and lifespan parameters.

2. Material and methods

2.1. Chemistry

The reactions were monitored by TLC carried out on Merck silica gel (60 F_{254}) by using UV light as visualizing agent and 5% vanillin in 10% H_2SO_4 and heat as developing agents. Baker silica gel (particle size 0.040–0.063 mm) was used for flash chromatography. Proton nuclear magnetic resonance spectra (^{1}H NMR) were obtained at 300 MHz on a Varian Gemini NMR and at 400 or 200 MHz on Bruker DPX 400 and DPX 200 spectrometers. Spectra were recorded in $CDCl_3$ solutions. Chemical shifts are reported in ppm, referenced to tetramethylsilane (TMS) as the external reference. Hydrogen coupling patterns are described as singlet (s), doublet (d), double doublet (dd), double triplet (dt), double double doublet (ddd), triplet (t), quintet (qui), sextet (sex), multiplet (m), and broad singlet (bs). Coupling constants (*J*) are reported in Hertz. Carbon-13 nuclear magnetic resonance spectra (^{13}C NMR) were obtained at 75 MHz on a Varian Gemini NMR and at 100 or 50 MHz on Bruker DPX 400 and DPX 200 spectrometers respectively. Chemical shifts are reported in ppm, referenced to the solvent peak of $CDCl_3$. Low-resolution mass spectra were obtained with a Shimadzu GC-MS-QP2010 mass spectrometer.

2.1.1. General procedure for the synthesis of 7-chloro-4-(organylsulfenyl)quinolines **3a–h**

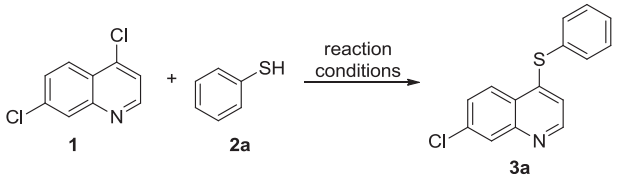
To a round-bottomed flask containing 4,7-dichloroquinoline (0.3 mmol) and DMSO (1.0 mL) was added the appropriated organylthiol (0.3 mmol). The reaction mixture was stirred at room temperature for the time indicated in Table 2. After that, the solution was cooled at room temperature, diluted with ethyl acetate (15 mL), and washed with water (3 × 15 mL). The organic phase was separated, dried over $MgSO_4$, and concentrated under vacuum. The obtained products were purified by flash chromatography on silica gel using a mixture of ethyl acetate/hexane (20:80) as the eluent.

*2.1.1.1 7-Chloro-4-(phenylthio)quinoline (**3a**) [12].* Yield: 0.078 g (96%); white solid; mp 73–75 °C. ^{1}H NMR ($CDCl_3$, 200 MHz) δ = 8.54 (d, *J* = 5.0 Hz, 1H), 8.29 (d, *J* = 2.0 Hz, 1H), 8.20 (d, *J* = 8.9 Hz, 1H), 7.64–7.53 (m, 6H), 6.78 (d, *J* = 5.0 Hz, 1H). ^{13}C NMR ($CDCl_3$, 50 MHz) δ = 153.54, 147.63, 144.84, 137.47, 135.53, 130.54, 130.47, 128.44, 127.72, 126.72, 124.88, 124.04, 117.05. MS (relative intensity) *m*/*z*: 273 (38), 271 (100), 235 (54), 204 (8), 135 (15), 127 (8), 109 (6), 77 (8).

*2.1.1.2 7-Chloro-4-(m-tolylthio)quinoline (**3b**).* Yield: 0.074 g (87%); white solid; mp 38–39 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 8.55 (d, *J* = 4.8 Hz, 1H), 8.12 (d, *J* = 8.9 Hz, 1H), 8.06 (d, *J* = 2.0 Hz, 1H), 7.50 (dd, *J* = 8.9, 2.0 Hz, 1H), 7.40–7.29 (m, 4H), 6.72 (d, *J* = 4.8 Hz, 1H), 2.39 (s, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ = 150.37; 149.30; 147.99; 140.11; 135.86; 135.59; 132.39; 130.71; 129.87; 128.73; 128.48; 127.27; 124.83; 124.26; 117.68; 21.24. MS (relative intensity) *m*/*z*: 287 (37), 285 (100), 270 (37), 251 (7), 249 (25), 236 (9), 135 (13), 111 (11), 91 (13), 77 (5).

*2.1.1.3 7-Chloro-4-(4-methoxyphenylthio)quinoline (**3c**).* Yield: 0.065 g (72%); white solid; mp 133–135 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 8.52 (d, *J* = 4.8 Hz, 1H), 8.13 (d, *J* = 8.9 Hz, 1H), 8.05 (d, *J* = 1.7 Hz, 1H), 7.53–7.50 (m, 3H), 7.02 (d, *J* = 8.6 Hz, 2H), 6.63 (d, *J* = 4.8 Hz, 1H), 3.88 (s, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ = 161.25; 150.45; 150.36; 148.00, 137.53; 135.60; 128.77; 127.21; 124.70; 124.09; 118.69; 116.86; 115.77; 55.44. MS (relative intensity) *m*/*z*: 303 (37), 301 (100), 270 (9), 266 (7), 257 (5), 251 (33), 235 (11), 223 (24), 139 (10).

Table 1
Optimization of reaction conditions in the synthesis of 7-chloro-4-(phenylsulfenyl) quinoline **3a**.[a]



Entry	Solvent	Temperature (°C)	Time (min)	Isolated yield **3a** (%)
1	DMSO	100	120	98
2	DMSO	100	60	97
3	DMSO	100	15	96
4	DMSO	r.t.	15	96
5	EtOH	r.t.	15	85
6	Glycerol	r.t.	15	55
7	PEG-400	r.t.	15	55
8	MeCN	r.t.	15	75
9	Toluene	r.t.	15	15

[a] Reactions are performed with 4,7-dichloroquinoline **1** (0.3 mmol), benzenethiol **2a** (0.3 mmol) under air atmosphere.

Table 2
Variability in the synthesis of 7-chloro-4-(arylsulfenyl)quinoline.[a]

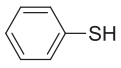
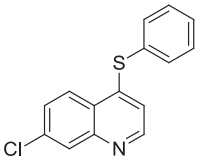
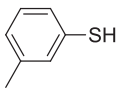
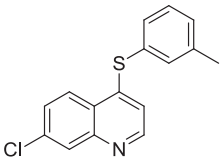
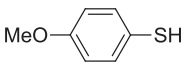
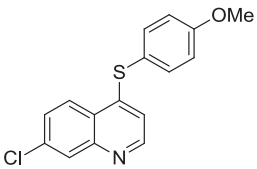
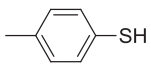
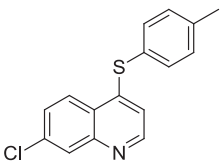
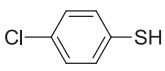
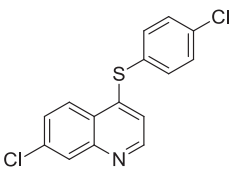
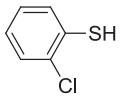
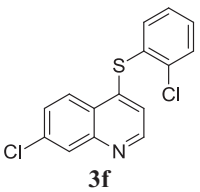
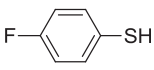
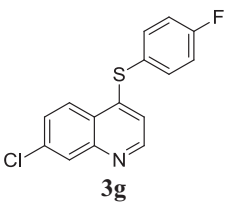
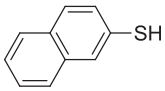
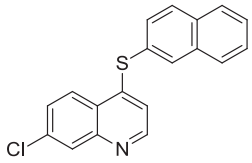
Entry	RSH	Time	Product	Yield (%)[b]
1	 **2a**	15 min	 **3a**	96
2	 **2b**	20 min	 **3b**	87
3	 **2c**	1.5 h	 **3c**	72
4	 **2d**	20 min	 **3d**	98
5	 **2e**	20 min	 **3e**	80
6	 **2f**	20 min	 **3f**	60
7	 **2g**	20 min	 **3g**	85

Table 2 (*continued*)

Entry	RSH	Time	Product	Yield (%)[b]
8	 **2h**	2 h[c]	 **3h**	80

[a] Reactions are performed with 4,7-dichloroquinoline **1** (0.3 mmol), organylthiol **2a**–**h** (0.3 mmol), in DMSO (1 mL) at room temperature under air atmosphere.
[b] Yields are given for isolated products.
[c] Reaction was performed at 70 °C.

*2.1.1.47-Chloro-4-(p-tolylthio)quinoline (**3d**).* [15] Yield: 0.084 g (98%); white solid; mp 115–117 °C. ^{1}H NMR ($CDCl_3$, 200 MHz) δ = 8.52 (d, J = 4.9 Hz, 1H), 8.17–8.10 (m, 2H), 7.56–7.46 (m, 3H), 7.31 (d, J = 7.9 Hz, 2H), 6.69 (d, J = 4.9 Hz, 1H), 2.44 (s, 3H). ^{13}C NMR ($CDCl_3$, 50 MHz) δ = 150.89; 149.68; 147.18, 140.61; 136.01; 135.62; 131.01; 128.23; 127.50; 124.78; 124.66; 124.12; 117.09; 21.36. MS (relative intensity) *m*/*z*: 287 (38), 285 (100), 272 (13), 270 (35), 250 (25), 236 (11), 235 (60), 207 (14), 135 (21), 127 (10), 111 (10), 91 (28), 77 (10).

*2.1.1.57-chloro-4-(4-chlorophenylthio)quinoline (**3e**).* Yield: 0.073 g (80%); white solid; mp 151–152 °C. ^{1}H NMR ($CDCl_3$, 200 MHz) δ = 8.58 (d, J = 4.8 Hz, 1H), 8.15–8.10 (m, 2H), 7.58–7.44 (m, 5H), 6.76 (d, J = 4.8 Hz, 1H). ^{13}C NMR ($CDCl_3$, 50 MHz) δ = 149.75; 149.28; 147.32; 136.49; 136.45; 136.25; 130.44; 128.34; 127.81; 127.30; 124.79; 124.22; 117.81. MS (relative intensity) *m*/*z*: 309 (13), 308 (13), 307 (65), 305 (100), 270 (52), 235 (99), 207 (19), 162 (12), 135 (44), 127 (21), 117 (18), 108 (22).

*2.1.1.67-Chloro-4-(2-chlorophenylthio)quinoline (**3f**).* Yield: 0.055 g (60%); white solid; mp 73–75 °C. ^{1}H NMR ($CDCl_3$, 200 MHz) δ = 8.60 (d, J = 4.8 Hz, 1H), 8.16–8.09 (m, 2H), 7.59–7.26 (m, 5H), 6.74 (d, J = 4.8 Hz, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ = 150.23; 148.22; 146.12; 138.46; 136.29; 135.90; 130.90; 130.74; 129.23; 128.80; 127.94; 127.66; 125.14; 124.77; 119.09. MS (relative intensity) *m*/*z*: 309 (6), 307 (32), 304 (44), 272 (36), 270 (100), 236 (15), 235 (79), 207 (12), 162 (6), 135 (26), 127 (11), 117 (12), 108 (14).

*2.1.1.77-Chloro-4-(4-fluorophenylthio)quinoline (**3g**).* Yield: 0.074 g (85%); white solid; mp 124–126 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 8.56 (d, J = 4.8 Hz, 1H), 8.10 (d, J = 8.9 Hz, 1H), 8.06 (d, J = 2.0 Hz, 1H), 7.59–7.55 (m, 2H), 7.52 (dd, J = 8.9, 2.0 Hz, 1H), 7.19 (t, J = 8.7 Hz, 2H), 6.66 (d, J = 4.8 Hz, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ = 163.82 (d, J^1 (C–F) = 251.7 Hz); 150.38; 148.98; 148.09; 137.67 (d, J^3 (C–F) = 8.5 Hz); 135.77; 128.86; 127.43; 124.69; 124.15; 124.10 (d, J^4 (C–F) = 3.6 Hz); 117.44 (d, J^2 (C–F) = 22.1 Hz); 117.41. MS (relative intensity) *m*/*z*: 292 (6), 291 (36), 290 (26), 289 (100), 254 (48), 222 (11), 207 (6), 162 (10), 135 (26), 127 (37), 57 (6).

*2.1.1.87-Chloro-4-(naphthalen-2-ylthio)quinoline (**3h**).* Yield: 0.077 g (80%); white solid; mp 66–67 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 8.53 (d, J = 4.8 Hz, 1H), 8.21 (d, J = 8.9 Hz, 1H), 8.16 (s, 1H), 8.09 (d, J = 1.9 Hz, 1H), 7.96–7.86 (m, 3H), 7.63–7.54 (m, 4H), 6.77 (d, J = 4.8 Hz, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ = 150.46; 148.15; 135.76; 135.42; 133.93; 133.43; 131.13; 129.95 (2C); 128.86; 127.90; 127.88; 127.60; 127.49; 127.06; 126.20; 124.96; 124.44; 118.16. MS (relative intensity) *m*/*z*: 323 (36), 321(100), 288 (23), 286 (49), 254 (19), 251 (5), 207 (17), 160 (20), 147 (5), 135 (15), 129 (23), 127 (19), 116 (10), 115 (38), 77 (9).

2.2. Caenorhabditis elegans strains and maintenance of the worms

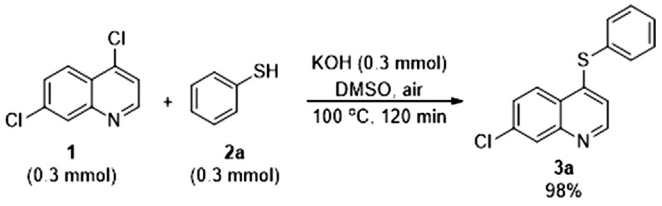
NGM plates seeded with live *Escherichia coli* OP50 bacteria were used for growth and maintenance of the worms, as previously described by Brenner [22]. The *C. elegans* strains Bristol N2 (wild type), CF1553 (muls84), GA800 (wuls151), TJ356 (zls356), CF1038 (*daf-16* [mu86]) were maintained and handled at 21 °C. Synchronous L1 population was obtained by isolating embryos from gravid hermaphrodites using bleaching solution (1% NaOCl; 2.4% NaOH), followed by floatation on a sucrose gradient to segregate eggs from dissolved worms and bacterial debris, accordingly to standard procedures previously described by Brenner [22].

2.3. Per se, protection and reversion protocols of treatment

1500 L1 worms previously synchronized were exposed for 30 min to the compounds in liquid media containing 0.5% NaCl and in absence of bacteria. L1 worms were chosen for these treatments because some reasons: The exposure at the L1 larval stage offers the possibility of to observe effects in the entire life of the worm and the treatment could be done in completely absence of *E. coli*, eliminating the possibility of any undesirable bacterial metabolization of the compounds. At the end of treatment, the worms were washed three times in NaCl to remove the compounds and soon after placed on NGM recovery plates seeded with *E. coli* OP50 (this procedure was made at the same mode before all the assays). The *per se*/protection/reversion protocols were assayed by counting alive worms in the plates 24 h post-treatment. A LD50 curve was obtained using concentrations that ranged from 1 μM to 1000 μM of the compounds, and then, nonlethal doses were chosen. The protection or reversion of the induced oxidative damage were assayed using paraquat (Gramoxone 200 ®) as pro-oxidant agent at the concentration of 500 μM for another 30 min. The pre or post exposure to the nonlethal concentrations of the compounds was followed by a post or anticipated by a pre, respectively, exposure to paraquat. Three additional washes were made before to proceed to all the assays. All the experiments were made in duplicates and repeated at least four times, as described by the Scheme 2.

2.4. Reproduction

In order to evaluate their reproduction, worms were individually transferred to new plates seeded with OP50 bacteria 48 h after treatments. Brood size was measured by monitoring the worms each 24 h during their fertile cycle. The mean eggs laid per day and



Scheme 1. Synthesis promoted by base of sulfenyl quinoline **3a**.

total brood size were quantified. All the experiments were made in duplicates and repeated at least five times.

2.5. Lifespan

48 h after the acute exposure described above, twenty worms per concentration were individually transferred to new plates seeded with *E. coli* OP50. The worms were monitored and transferred to new plates every day until the last one died. All the experiments were made in duplicates and repeated at least three times.

2.6. ROS measurement

The ROS levels were measured in the L1 and L4 larval stage. For L1 worms, right after the treatments, the animals washed to remove the compounds and then incubated for 1 h with 50 μM of H_2DCF-DA; then, worms were washed with 0.5% NaCl until the concentration of the dye decreased approximately a thousand times (three dilutions of 1:10). Worms were transferred to microplates and the fluorescence levels were measured for 30 min using a plate reader CHAMELEON™V Hidex Model 425-106, heated at 20 °C. For L4 worms, worms were treated at the L1 stage, which were deposited in NGM plates seeded with *E. coli*. 48 h after, the NGM plates were washed with M9 buffer and the worms were transferred to microtubes. The *E. coli* supernatant was washed until exhaustion (three dilutions of 1:10). Then, worms were concentrated to 100 μL with M9 buffer. 50 μL was incubated with 50 μM of H_2DCF-DA for 1 h, washed for dye removal and the ROS levels measured by the same manner as L1 worms. The remaining 50 μL of the sample was ultrasonicated for protein measurement, according to Bradford [30].

2.7. Fluorescence quantification

The GFP expressing strains (CF1553 [muls84] and GA800 [wuls154]) were submitted to the acute exposure as above described and transferred right after the end of the washes into 200 μL M9 buffer in a well of 96-well plate. Total GFP fluorescence was measured using 485 nm excitation and 530 nm emission filters. Overall GFP fluorescence of GFP-expressing strains was assayed using a plate reader CHAMELEON™V Hidex Model 425-106. All experiments were made in duplicates and repeated at least five times.

2.8. Epifluorescence microscopy

For each slide, at least 30 treated worms were mounted on 2% agarose pads and anaesthetized with few drops of levamisole 22.5 μM. Fluorescence observations were performed under epifluorescence microscopy; housed in air-conditioned rooms (20–22 °C) for image acquisitions and scoring the expression levels.

2.9. Statistics

All the graphics were generated with GraphPad Prism (GraphPad Software Inc.). Dose-response curves were generated in order to obtain the LD_{50} of each compound. To analyze the ROS measures and lifespan, a Repeated Measures ANOVA analysis and the Tukey post-hoc were applied. A one-way ANOVA was applied for all the other assays, followed by the Tukey post-hoc. All the p values less than 0.05 were considered statistically significant. All the values expressed in percentage (%) were normalized assuming that control values as 100%. In all figures bars represent the SEM.

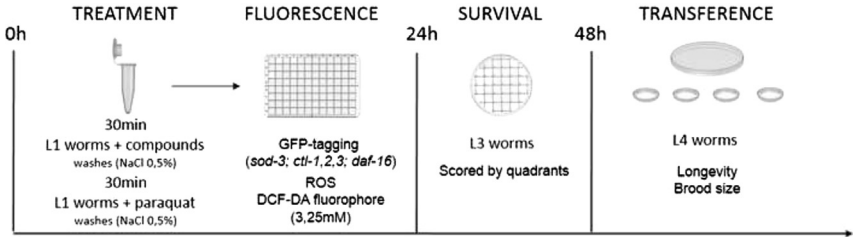
3. Results

3.1. Synthesis of sulfenyl quinolines

Initially, our studies were focused on the synthesis of 7-chloro-4-(phenylsulfenyl)quinoline **3a**. Recently, our group described the synthesis of 4-arylselanyl-7-chloroquinolines by reaction of 4,7-dichloroquinoline with diaryl diselenides using KOH as base, DMSO as solvent at 100 °C under air atmosphere [31]. Extending this reaction condition for the reaction of 4,7-dichloroquinoline **1** with benzenethiol **2a**, the desired product **3a** was obtained in excellent yield (Scheme 1).

Notably, when we performed this reaction in the absence of KOH, the desired product **3a** was obtained in similar yield after 120 min (Table 1, entry 1). The same results were also observed in reactions decreasing the reaction time to 60 and 15 min (Table 1, entries 2 and 3). When the reaction of quinoline **1** and benzenethiol **2a** was performed at room temperature for 15 min, an excellent result was achieved (Table 1, entry 4). We observed that the nature of the solvent was critical for obtain the desired product **3a** in high yields and short reaction time (Table 1, entries 4–9). As shown in Table 1, reactions employing different solvents, such as EtOH, glycerol, PEG-400, MeCN and toluene, gave moderated or only a traces of product **3a** (Table 1, entries 4–9). Thus, analysis of the results showed in Table 1 indicated that the best reaction conditions were the use of 4,7-dichloroquinoline **1** (0.3 mmol), benzenethiol **2a** (0.3 mmol) using DMSO as solvent at room temperature under air atmosphere for 15 min.

After that, the variability of our methodology reacting 4,7-dichloroquinoline **1** with other organylthiols **2b**–**h** was evaluated



Scheme 2. Schematic line of the treatments and assays.

(Table 2). The results showed in Table 2 reveal that the reaction worked well with a range of substituted arylsulfides containing electron-donating (EDG) and electron-withdrawing groups (EWG), affording good yields of the desired products (Table 2, entries 1–7). We observed that steric effect had a little influence on the product formation and reaction using 2-naphthylmercaptan gave the desired product **3h** in 80% yield, however at 70 °C and in a longer reaction time compared with other examples (Table 2; entry 8).

3.2. The compounds depicted different LD_{50} depending on the substitutions of the thioquinoline ring

As showed in Fig. 1A, we found different LD_{50} values to the compounds, indicating that the toxicity of the thioquinolines is directly associated with the different substituents. In decrescent order of concentration: **3h** (2500 μM) > **3d** (1700 μM) > **3e** (520 μM) > **3c** (230 μM) > **3b** (180 μM) > **3a** (120 μM) > **3f** (45 μM) > **3g** (18 μM). The nonlethal concentrations were chosen taking into account the lowest value of LD50 found (**3g**–18 μM). The chosen concentration was 10 μM and no toxicity in the survival assay was verified at this concentration in all thioquinolines tested, as showed in Fig. 1B.

3.3. Compounds protected against the induced oxidative damage inflicted by paraquat

Searching for the best use of the compounds as antioxidant agents, we choose to perform protection and reversion protocols against the induced oxidative damage inflicted by paraquat (500 μM). We found that the compounds show their better potential as protectors (Fig. 2A) by preventing the mortality of the worms acutely exposed to paraquat. Worms treated only with paraquat showed approximately 50% of mortality, whereas five compounds showed reduced mortality levels around 34-26% when pre-administered previous to paraquat. The reversion protocol was not as efficient, as only **3h** depicted a significant reduction on the mortality induced by paraquat (Fig. 2B).

3.4. The compounds protect against paraquat-induced lifespan toxicity

Fig. 3A and B show that six compounds did not alter worms lifespan. Compounds **3f** and **3h** increased the mean time of life ($p < 0.0001$ compared with the control group). Paraquat caused toxic effects, resulting in reduced lifespan of the exposed worms, as demonstrated in Fig. 3C and D ($p < 0.0001$ when compared to the control group). Three of the compounds (**3c**, **3f** and **3h**) were able to protect the worms against the reduction in lifespan caused by paraquat exposure ($p < 0.0001$ compared to paraquat group).

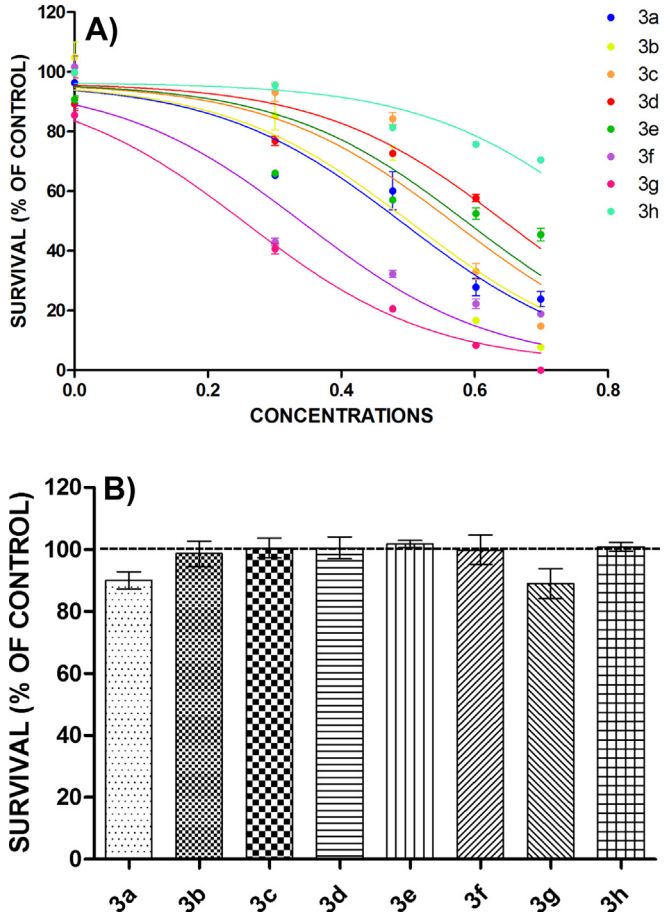


Fig. 1. *Per se* effect of thioquinolines: A) Dose–response curve for LD50% (Lethal Dose 50%), B) non-lethal concentration of 10 μM on survival. Data are expressed as mean ± SEM.

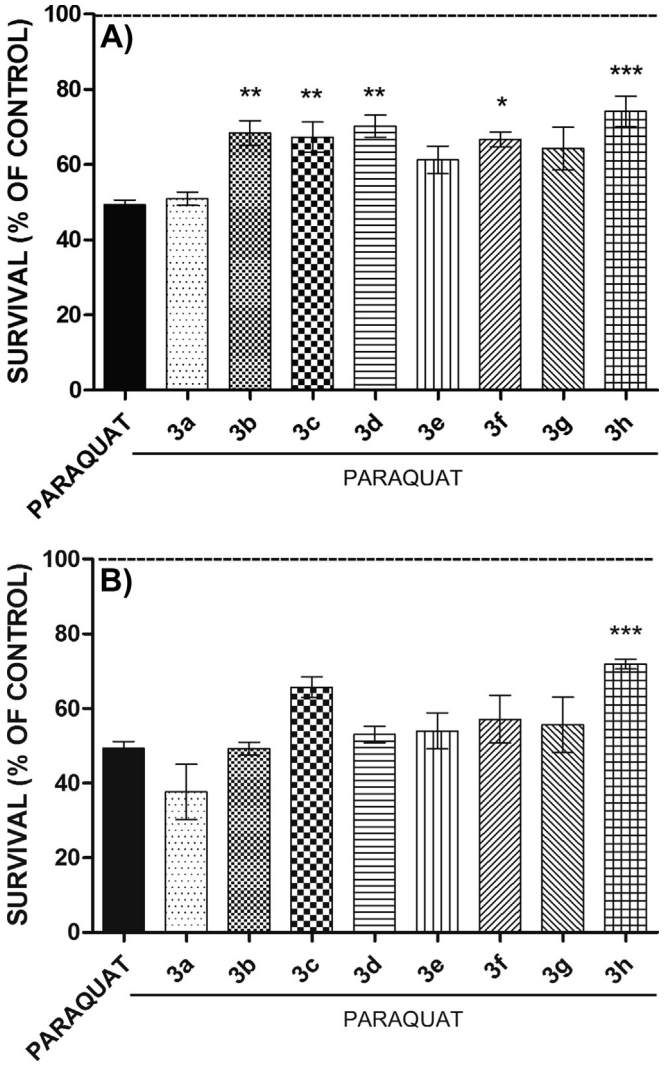


Fig. 2. Protection and reversion treatments with thioquinolines: A) Protection, B) reversion. Data are expressed as mean ± SEM. The control group was considered as 100%. *, ** and *** indicate, respectively, $p < 0.05$, $p < 0.01$ or $p < 0.0001$ compared to the paraquat group.

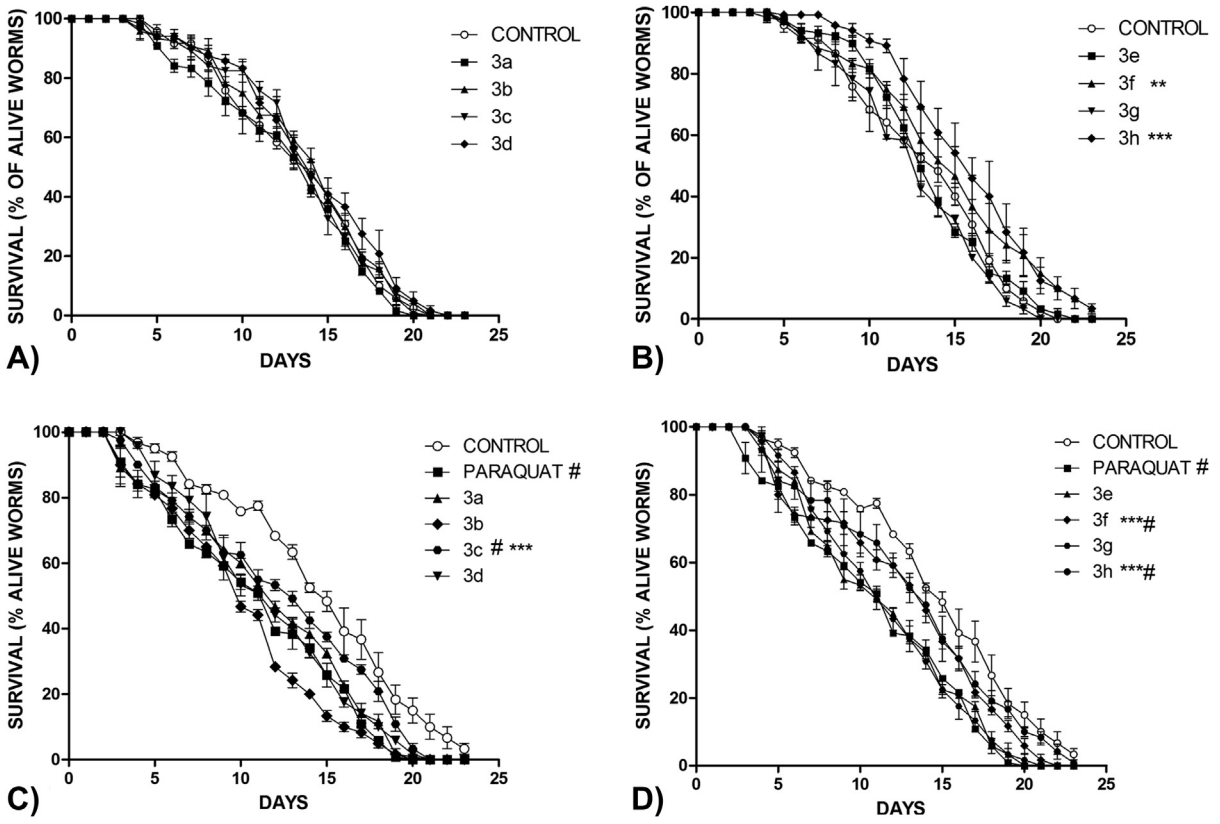


Fig. 3. Longevity after per se or reversion treatment with thioquinolines: A and B) *Per se* C and D) Protection. Data are expressed as mean ± SEM. Data were normalized to 100%. # indicates significant difference ($p < 0.05$) compared to the control group. * indicates significant difference compared to the paraquat group.

3.5The compounds protect against the paraquat-induced reproductive toxicity

All the compounds did not shown any *per se* effects on reproduction, as observed by brood size assay (Fig. 4A). Fig. 4B shows that the group treated only with paraquat depicted significant reduction in the egg-laying ($p < 0.0001$ when compared to the control group). **3d** was able to partially protect the reduction in brood size caused by paraquat ($p < 0.01$ compared to paraquat group) whereas two other compounds (**3c** and **3h**) fully protected the worms, depicting number of eggs indistinguishable from control group (Fig. 4B).

3.6. The ROS levels in worms were altered in the per se and protection protocols

To determine whether the ROS formation caused by paraquat could be prevented by the compounds, we used H_2DCF-DA, which is de-esterified to H_2DCF and then oxidized to the DCF fluorophore in the presence of free radicals. Fig. 5A shows that all compounds caused increase in ROS formation in L1 worms; nevertheless, in these same worms, 48 after the treatment (Fig. 5B), ROS levels were indistinguishable (**3c**) or lower (**3b** and **3d-3h**) than the levels found in the controls. **3a** compound-treated group remained with higher ROS levels even 48 h after the treatment. Paraquat- treatment and compounds/paraquat treatment depicted increased ROS levels in L4 worms when compared to control ($p < 0.0001$, Fig. 5C); however, two groups of treated worms (**3c** and **3h**) showed significantly reduced levels of ROS when compared to paraquat-exposed worms ($p < 0.0001$).

3.7. Levels of mitochondrial superoxide dismutase (SOD-3) and catalase (CTL-1, CTL-2, CTL-3) are increased in worms pre-treated with the compounds

High levels of ROS can lead to an oxidative stress state. In a healthy organism, some enzymes play a detoxifying role. The *sod-3* gene encodes the major isoform of mitochondrial superoxide dismutase, an important antioxidant enzyme that catalyzes the dismutation of superoxide to hydrogen peroxide. Thereafter, H_2O_2 is converted to molecular oxygen and water by catalase, other antioxidant enzyme. We found that the treatment with **3c**, **3e** and **3h** compounds were able to significantly increase the levels of both SOD-3 and CTL-1,2 and 3, isoforms, whereas **3d** was capable to induce only SOD-3 expression and **3g** only shows ability to up-regulate the CTL isoforms, as showed in Fig. 6A and B, respectively.

3.8. The protective effect of the compounds is dependent of DAF-16, a homolog of the human transcription factor FOXO

Searching for a putative molecular target of the thioquinolines, we investigated the subcellular localization of DAF-16. The transcription factor DAF-16 is the worm homolog of human FOXO. This transcriptional factor belongs to the insulin/IGF-like signaling, which is the central determinant of the endocrine control of stress response, aging, fat metabolism, fertility, and diapause in *C. elegans*. In the worms pre-treated with the compounds, we observed a significant change in DAF-16 localization for **3c**, **3d** and **3f**–**h** compounds, inducing its nuclear migration and thus, its activation ($p < 0.0001$, Fig. 7A).

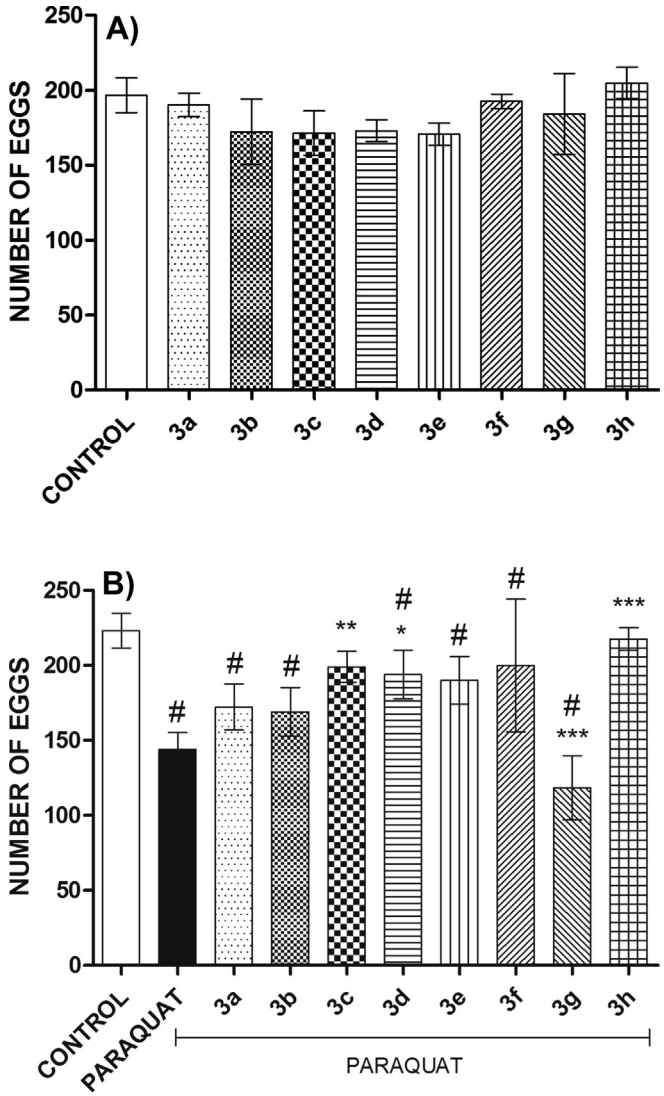


Fig. 4. Brood size in *per se* and reversion treatment with thioquinolines: A) *Per se*, B) Reversion. Data are expressed as mean ± SEM. # indicates significant difference ($p < 0.05$) compared the control group. * indicates significant difference compared to the paraquat group ($p < 0.05$).

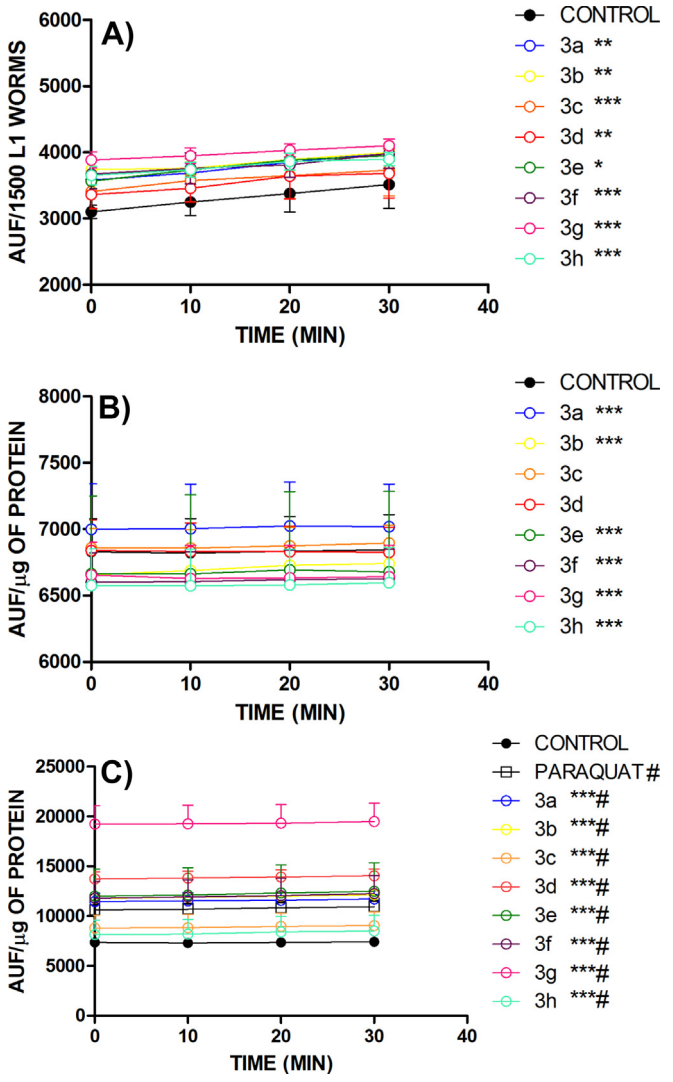


Fig. 5. ROS levels measured with H_2DCF-DA probe in *per se* and protection treatment with thioquinolines: A) *Per se* in N2 L1 animals, B) *Per se* in N2 L4 (48 after the treatment) animals C) Compounds/paraquat in L4 (48 h after the treatment) animals. Arbitrary Units of Fluorescence/µg of protein. Data are expressed as mean ± SEM. *, ** or *** indicate difference when compared to paraquat group. # indicates difference when compared to control group.

The compounds caused mortality *per se* in *daf-16* mutants (Fig. 7B). We also found that the protection against the oxidative damage conferred by the pre-treatment with the thioquinolines in the survival assay was completely lost in absence of the *daf-16* gene, as depicted in Fig. 7C. The elevated ROS formation induced by the compounds *per se* was significantly higher in the *daf-16* mutant animals than the observed in N2 worms (Fig. 7D and Table 3). Besides, 48 h after, these ROS levels remained significantly elevated but without any difference in comparison to N2 animals, as showed in Fig. 7E and Table 3. The post-treatment with paraquat in L4 animals significantly induced higher ROS formation in *daf-16* animals than in N2 (Fig. 7F. and Table 3). Besides, none of the compounds were able to prevent the induced ROS formation in the *daf-16* mutants (Fig. 7E).

4. Discussion

In this study we investigated the toxicity and pharmacological potential of a series of novel compounds based on the sulfur containing quinoline compounds in *C. elegans*. We found that these compounds were able to protect against the oxidative damage inflicted by paraquat in several parameters and demonstrated that this ability is possibly due to a hormetic response against a small oxidative damage induced by the thioquinolines. This response could be, at least in part, mediated by ROS formation, which in turn activates the FOXO homolog DAF-16 stress response, up-regulating the levels of SOD-3 and CTL-1,2,3, partially neutralizing the ROS formation induced by paraquat. This hormetic response could protect against the subsequent oxidative damage induced by the powerful prooxidant paraquat, an agent that affects survival, reproduction and longevity parameters in *C. elegans*.

Focusing at the safe use of the thioquinolines, we have primarily assessed their toxicological profile. Organochalcogens toxicity has been linked to the stability of the carbon-chalcogen bond [7], which could explain the behavior of the thioquinolines studied in this work. Compound **3g** had a higher toxicity (18 µM) probably because this compound has a fluor atom, a weak deactivating with high electronegativity, which facilitates reaction with another biomolecules. On the other hand, compound **3h**, which has the neutral

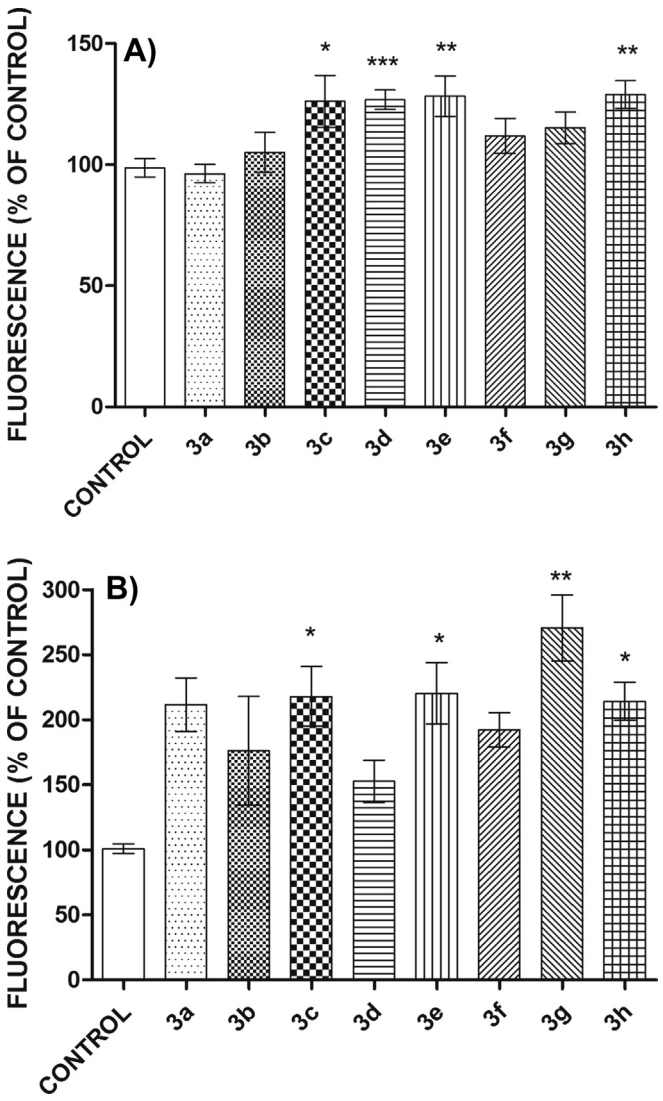


Fig. 6. Antioxidant enzymes levels following thioquinolines treatment A) SOD-3:GFP, B) CTL-1,2,3 :GFP. Data are expressed as mean ± SEM. *, ** and *** indicate, respectively, $p < 0.05$, $p < 0.01$ or $p < 0.0001$ compared to the control group.

and stable naphthalene heterocycle bond to the chalcogen, presented the lowest toxicity (2500 μM). The pairs **3b**/**3d** and **3e**/**3f** are constitutional isomers. As both **3d** and **3e** showed lower toxicity of each pair and have activating substituents that guide the reaction to the orto and para position, we believe that the para position is blocked by the calchogen atom and consequently the formation of a possible toxic product is diminished. **3b** and **3f** have activating substituents that guide the reaction to the same positions and, in that case, they are free to react. Compound **3c** has a methoxy substituent that could guide the reaction to the position blocked by the chalcogen, but in this case the high electron donating-ability of this group, in comparison with the methyl group, seems to contribute to this effect, taking into account its elevated toxicity when compared to the others (230 μM). **3a** has high toxicity in comparison to the others thioquinolines (120 μM), probably because of the absence of substituents that could stabilize the structure. As the lowest lethal concentration was 18 μM (**3g**), we decided to test a nonlethal concentration of 10 μM for all the compounds. We verified that, at this concentration, all the thioquinolines induced ROS formation in recently L1-treated worms (Fig. 5A) without causing any mortality (Fig. 1B). This is consistent with the lower ROS levels found 48 h after thioquinolies treatment (compounds **3b**–**h**) (Fig. 5B). It has been reported that primaquine, an anti-malarial drug based on the quinoline ring, has pro-oxidant activity against *Plasmodium falciparum* in the red blood cells. Primaquine can generate hydroxyl radicals from oxidation of pyridine nucleotides, such as NADH and NADPH [32]. This can explain the elevated amounts of ROS in the worms following thioquinolines treatment, *per se*. Interestingly, we verified the absence of toxic effects following thioquinolines treatment on longevity (Fig. 3A and B)and reproduction endpoints (Fig. 4B). In fact, we have found that compounds **3f** and **3h** prolonged lifespan of treated worms, *per se*. This effect can be attributed to the theory that small ROS formation could have beneficial effects in some ROS-associated situations, such as aging or even physical exercise, where there is an imbalance of antioxidant/prooxidant homeostasis [33–36], a mechanism known by hormesis. More studies are required to clarify these point, but we suggest a possibly involvement of DAF-16 translocation to the nucleus, since that the superexpression of this one can extend the longevity of *C. elegans* in non-stress conditions [40].

Paraquat toxicity mechanisms involve the generation of the superoxide anion, which can lead to the formation of more toxic reactive oxygen species, such as hydrogen peroxide and hydroxyl radical; and the oxidation of the cellular NADPH, the major source of reducing equivalents for the intracellular reduction of paraquat, which results in the disruption of important NADPH, required in biochemical processes [37]. In our study we could observe that paraquat reduced significantly the survival (Fig. 2A and B) the longevity (Fig. 3C and D) and reproduction (Fig. 4B) in the acute treatment. Besides, the ROS levels were significantly higher than in the non-treated worms (Fig. 5C and D). Thus, our findings suggest that ROS formation induced by paraquat can negatively affect survival, longevity and reproduction in *C. elegans*, which is in agreement to previous studies [38,39]. Notably, ROS formation induced by the thioquinolines did not affect any of these parameters.

In agreement to the hormetic response theory, five of the compounds (**3b**–**d**, **3f** and **3h**) protected the worms against the injury caused by paraquat on survival, and only three of these (**3c**, **3f** and **3h**) were able to offer protection against the reduced lifespan induced by paraquat. We have tested whether the thioquinolines could reverse the oxidative damage, however only **3h** was able to increase survival after paraquat exposure (**3h** in Fig. 2B). This is an additional finding that supports the hormetic mechanism provided by the compounds, as they could not decrease mortality once the damage-induced by paraquat is already present.

In *C. elegans*, brood size represents the reproductive capacity and can be used as a parameter of toxicity [38]. **3c** and **3h** were able to completely recover from paraquat-induced reproductive toxicity (Fig. 4B). We also observed that paraquat toxicity in *C. elegans* could be associated to high and persistent ROS formation (Fig. 5C and D), whereas **3c** and **3h** pre-treatment prevented the increased ROS levels in those two treated groups (Fig. 5D).

Antioxidant compounds that enhance stress resistance without prolonging lifespan of *C. elegans*, as **3b** and **3d**, have been described in the literature [41,42], demonstrating again the controversial role of the ROS in the aging process. On the other hand **3c**, **3f** and **3h** were able to protect from the oxidative damage-induced by paraquat in survival and lifespan endpoints, in accordance to the hypothesis that the oxidative state in *C. elegans* could modulate these two parameters at the same time [43].

In addition, the putative hormetic thioquinolines response may act by modulating some intracellular process that modulate antioxidant defenses, so we further investigated whether thioquinolines caused the translocation of the transcription factor DAF-16 into the nucleus. Four thioquinolines that protected against paraquat-induced mortality and reduced ROS levels induced by this

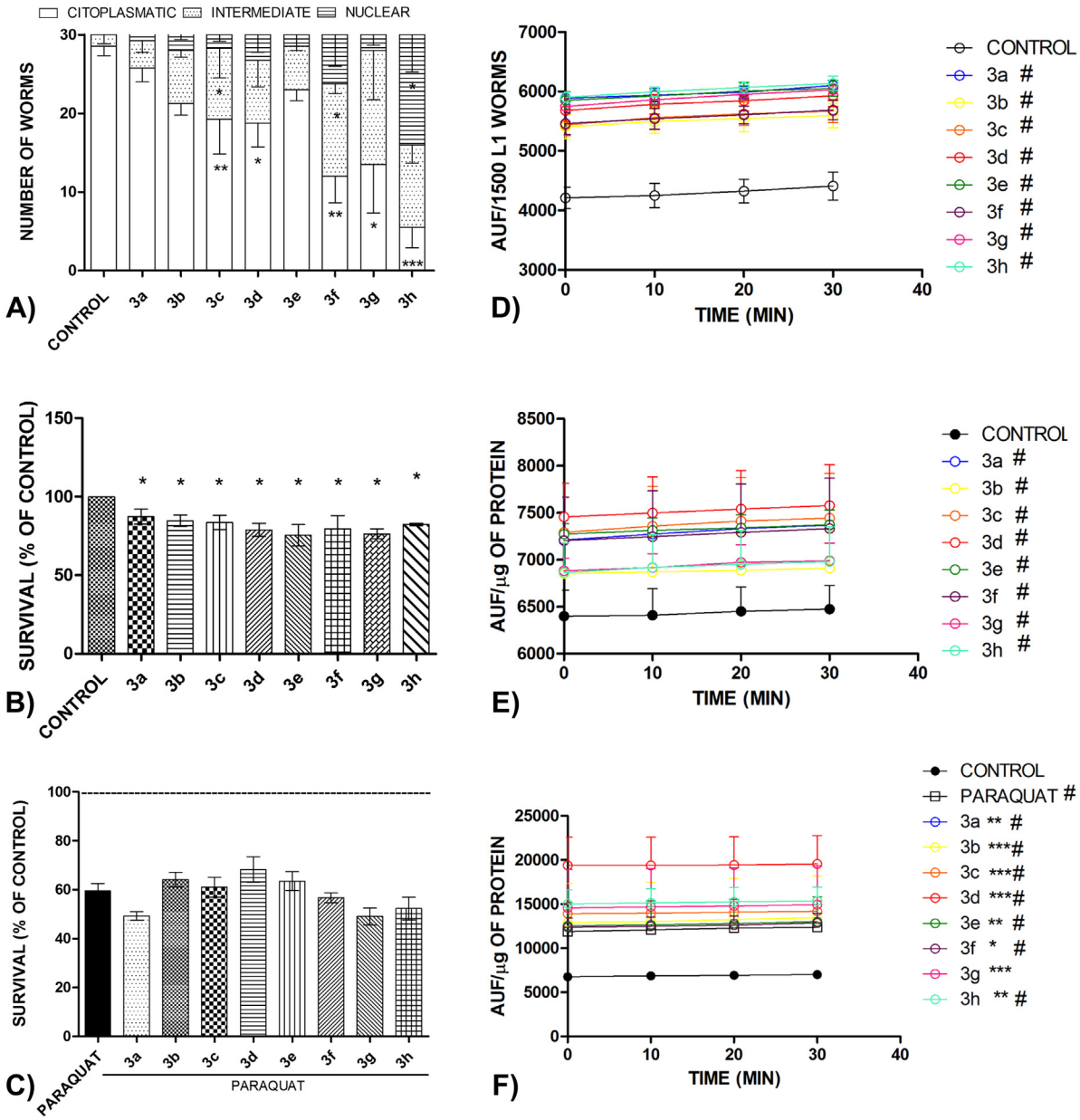


Fig. 7. Thioquinolines modulate DAF-16 pathway *per se*: A) Subcellular localization of DAF-16 was considered in three levels: cytosolic, intermediate and nuclear, 30 worms per group were visualized and scored. B) Thioquinolines effects on survival of knockdown mutants lacking *daf-16*. C) Thioquinolines/paraquat treatment effect above survival of mutants lacking *daf-16*. D) ROS levels of L1 *daf-16* mutants soon after the thioquinolines treatment. E) ROS levels of L4 *daf-16* mutants 48 h after the thioquinolines treatment. F) ROS levels of L4 *daf-16* mutants 48 h after the thioquinolines/paraquat treatment. Arbitrary units of fluorescence/µg of protein. Data are expressed as mean ± SEM. *, ** or *** indicates difference when compared to paraquat group. # indicates difference when compared to control group. *, ** and *** indicate, respectively, $p < 0.05$, $p < 0.01$ or $p < 0.0001$ compared to the control group.

pesticide (**3c**, **3d**, **3f**, and **3h**) were able to modulate the subcellular localization of DAF-16, increasing its translocation to the nucleus (Fig. 7A). Furthermore, **3c** and **3h** up-regulated the expression of both SOD-3::GFP and CTL-1,2,3::GFP (Fig. 6A and B), classical targets of DAF-16-mediated stress response [44,45], possibly as a consequence of its migration to the nucleus. To confirm whether thioquinolines were indeed modulating the IGF-1 pathway at the DAF-16 level, we used a knockdown strain for *daf-16* (mu86) and verified that the compounds induced mortality, *per se* (Fig. 7B). Besides that, the protection against paraquat in the survival parameter was completely lost (Fig. 7C). We also verified that these mutants had significantly more ROS levels than wild-type animals soon after the thioquinolines treatment and 48 h after (Fig. 7D and E and Table 3 for comparison between strains) Furthermore, none of the compounds were able to prevent the paraquat-induced ROS formation in the absence of *daf-16* (Fig. 7F). Considering such observations we concluded that DAF-16 plays a vital role in the thioquinolines response, probably by detoxifying the ROS generated by them and, furthermore, protecting worms against paraquat toxicity, which could explain the *per se* toxicity and the absence of protection in *daf-16* mutant. Many studies have demonstrated that DAF-16 acts in the hormetic response against many toxicants by activating mechanisms that repair damage and protect the organism from further injury. This oxidative-stress response has been associated with the up-regulation of the mitochondrial superoxide dismutase SOD-3 (leading into account that mitochondria is the

Table 3
Comparison between the ROS levels in N2 and CF1038 strains.

Compound (N2×CF1038)	Thioquinolines L1	Thioquinolines L4	Thioquinolines/ paraquat L4
Control	–	–	–
Paraquat	–	–	*
3a	***	–	–
3b	**	–	–
3c	**	–	*
3d	**	–	–
3e	***	–	–
3f	**	–	–
3g	***	–	–
3h	***	–	*

Significantly difference by *t*-student test, where *, ** and *** indicates, respectively, $p < 0.05$, $p < 0.01$ and $p < 0.001$.

major ROS source within the cell) and catalase, the enzyme that acts on the H_2O_2 formed by SOD [46–48].

In summary, our data suggest that **3c** and **3h** both improved worms survival, longevity, brood size and diminished ROS formation in an oxidative stress background induced by paraquat probably through the translocation of DAF-16 to the nucleus, thus increasing the transcription of SOD-3 and CTL-1,2,3. Our acute and low dose exposure protocol points out to **3h** as the most promising molecule. We observed that **3c** presented the same protector effects as **3h** on paraquat-induced damage, however it has shown higher toxicity, as indicated by the LD_{50} (Fig. 1A). This suggests a larger and safer therapeutic window to **3h**. In turn, these molecules can neutralize ROS generated in response to paraquat and attenuate the damage caused by this toxicant in *C. elegans*. Interestingly the pre-treatment with **3d** and **3g** induces the translocation of DAF-16 into the nucleus, however **3d** increased only SOD-3 expression and **3g** only CTL-1,2,3. These two compounds were not that effective to protect the worms from paraquat oxidative damage on survival, longevity, brood size and excessive paraquat-induced ROS-formation, suggesting that the increased expression of both enzymes are necessary to enhance stress resistance against this pesticide.

5. Conclusion

In summary, we have demonstrated the efficient synthesis and biological properties in *C. elegans* of a range of 4-organylsulfenyl-7-chloroquinolines. This class of compounds have been easily synthesized in high yields by direct reaction of 4,7-dichloroquinoline with organylthiols under mild reaction conditions and tolerates a range of substituents in the organylsulfenyl moiety. The obtained results revealed that the novel thioquinolines depicted a structure-dependent effect regarding their toxicity and pharmacology. The acute treatment with a small and non-lethal concentration of **3c** and **3h** were able to reduce paraquat-induced ROS formation in the worm body probably due to a hormetic response that in turns up-regulated the expression of the antioxidant enzymes SOD-3 and CTL-1,2,3 by modulating the translocation of the transcription factor DAF-16 into the nucleus. Consequently, we observed restoration of the endpoints analyzed *in vivo* in *C. elegans*: survival, brood size and longevity. Our findings illustrated the utility of the worm model in elucidating protective and toxic mechanism as well as for the identification of pharmacological targets for drug development.

Acknowledgments

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