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Phenazine derivatives cause proteotoxicity and stress in *C. elegans*

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HIGHLIGHTS

- Phenazines cause α-synuclein and polyglutamine-induced protein misfolding in *C. elegans*.
- These compounds exacerbate α-synuclein-induced dopaminergic neurodegeneration in *C. elegans*.
- Addition of anti-oxidant fails to attenuate the toxic phenotypes caused by phenazines.

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ABSTRACT

It is widely recognized that bacterial metabolites have toxic effects in animal systems. Phenazines are a common bacterial metabolite within the redox-active exotoxin class. These compounds have been shown to be toxic to the soil invertebrate *Caenorhabditis elegans* with the capability of causing oxidative stress and lethality. Here we report that chronic, low-level exposure to three separate phenazine molecules (phenazine-1-carboxylic acid, pyocyanin and 1-hydroxyphenazine) upregulated ER stress response and enhanced expression of a superoxide dismutase reporter in vivo. Exposure to these molecules also increased protein misfolding of polyglutamine and α-synuclein in the bodywall muscle cells of *C. elegans*. Exposure of worms to these phenazines caused additional sensitivity in dopamine neurons expressing wild-type α-synuclein, indicating a possible defect in protein homeostasis. The addition of an anti-oxidant failed to rescue the neurotoxic and protein aggregation phenotypes caused by these compounds. Thus, increased production of superoxide radicals that occurs in whole animals in response to these phenazines appears independent from the toxicity phenotype observed. Collectively, these data provide cause for further consideration of the neurodegenerative impact of phenazines.

1. Introduction

Phenazines are a class of nitrogen containing heterocyclic compounds that display a range of biological effects, including anti-bacterial and anti-tumor activities. The parent compound, dibenzopyrazine, or phenazine, has a molecular structure of $C_{12}H_8N_2$. Phenazine derivatives are produced by several bacterial genera including *Nocardia*, *Streptomyces*, and *Pseudomonas* [1]. They have been identified as virulence factors in plants, animal models, and humans [2,3].

The most commonly studied phenazine derivatives are pyocyanin (PCN), phenazine-1-carboxylic acid (PCA), and 1-hydroxyphenazine (1-HP). Among these, PCN and 1-HP are unique to *Pseudomonas aeruginosa* [4]. 1-HP is a degradation product of PCN, which is produced from the precursor PCA. These compounds exert their effects by increasing formation of reactive oxygen species (ROS), which can then affect cellular functions [5].

Caenorhabditis elegans, a free-living soil nematode has been used as a pathogenesis model to test phenazine toxicity. PCN is the most extensively characterized phenazine in *C. elegans*; it is secreted from *P. aeruginosa* and kills worms by causing oxidative stress [6]. It also causes infection in *Drosophila*, mice, and plants [3,6–8]. PCN plays an important role in iron metabolism and redox cycling in human epithelial cells where it depletes cellular ATP and alters mitochondrial electron transport [9,10]. Further studies report that catalase and glutathione levels are compromised in the presence of PCN [11]. PCA and 1-HP have also been reported to kill *C. elegans* in a matter of hours at high concentrations [12].

In mammalian astrocytoma cells, PCN and 1-HP inhibit mitochondrial respiration [11]. Mitochondrial dysfunction plays an important role in the neurodegenerative disorders Parkinson's disease (PD), and Huntington's disease (HD) [13,14]. These diseases are also associated with aggregation of intracellular toxic

Abbreviations: PD, Parkinson's disease; HD, Huntington's disease; PCA, phenazine-1-carboxylic acid; PCN, pyocyanin; 1-HP, 1-hydroxyphenazine; NAC, N-acetyl cysteine; UPS, ubiquitin proteasome system; UPR, unfolded protein response.

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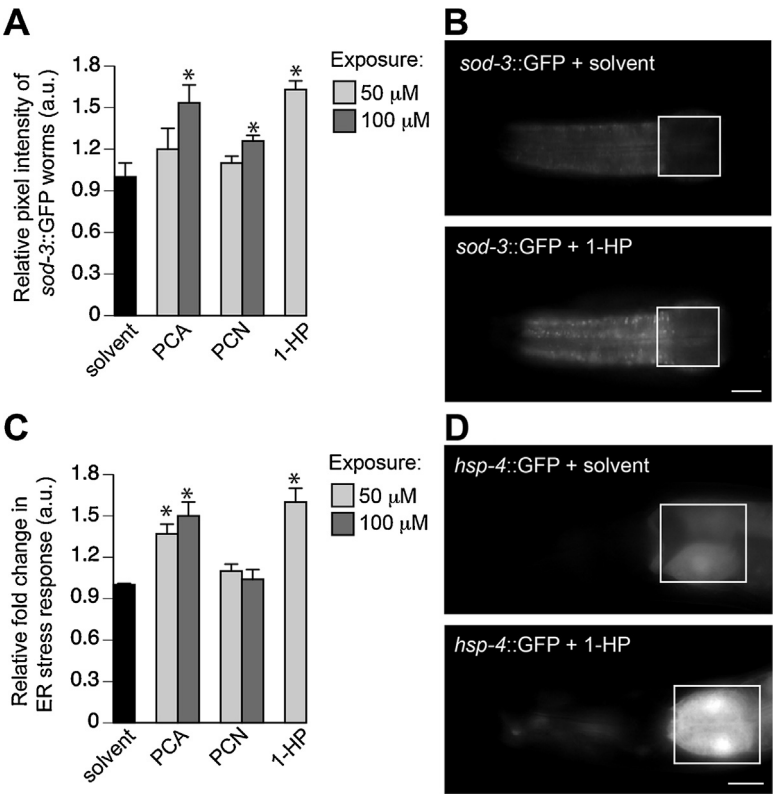

Fig. 1. Phenazine derivatives cause increased *sod*-3 expression and ER stress response in *C. elegans*. (A) Upregulation of *sod-3*::GFP expression, an indicator of oxidative stress, occurred following exposure to PCA, PCN, and 1-HP, compared with worms exposed to solvent only at day 4. This was quantitated using pixel intensities, as described in B. Values are the mean ± SD of 3 experiments where 30 animals were analyzed per replicate ('*' represents significance between individual treatments and solvent control where $P < 0.05$ by one-way ANOVA). Values were normalized to the untreated solvent control. (B) Representative images for phenazine treatments described in A, where pixel intensities were measured in an anatomically-invariant 100 × 100 μm region at the anterior bulb of the pharynx (white box). Scale bar, 50 μm. (C) PCA and 1-HP significantly increased the ER stress response (*hsp-4*::GFP expression) compared with solvent control at day 4 ($P < 0.05$; one-way ANOVA; $n = 3$ experiments; 30 worms per replicate). Values were normalized to untreated *hsp-4*::GFP control. (D) Representative images of worms for phenazine treatments described in C where GFP pixel intensities were measured as in B. Scale bar, 50 μm.

proteins. Growing evidence for phenazines affecting mitochondrial activity led us to hypothesize that phenazine derivatives might impact proteostasis and contribute to neurodegeneration following chronic exposure to non-lethal doses. To investigate this we used well-characterized *C. elegans* assays that have proven predictive in discerning both genes and compounds that exhibit activities translatable to mammals [15,16].

2. Materials and methods

PCN (Cayman Chemicals), 1-HP (TCI America) and PCA (Princeton Biomedical Research) were dissolved in DMSO (<2%) and mixed in nematode growth medium (NGM) before pouring in small Petri dishes. Concentrations in a range of ~100–300 μM are lethal to *C. elegans* depending on specific molecule [12], therefore 50 μM and 100 μM final concentrations of each phenazine compound were tested for chronic exposure. For 1-HP, 100 μM was lethal to the worms, thus only 50 μM was used.

Nematodes were maintained using standard procedures. The following strains were obtained from the *Caenorhabditis* Genetics Center: KN259 [*huIs33*{*sod-3*::GFP + pRF4(*rol*-6(*su1006*)}] and SJ4005 [*zcIs4*(*hsp-4*::GFP)]. BY250 [*vtIs7*($P_{dat\text{-}1}$::GFP)] and AM141 [*rmIs133*($P_{unc\text{-}54}$::Q40::YFP)] were gifts from Randy Blakely (Vanderbilt) and Rick Morimoto (Northwestern).

C. elegans hermaphrodites have 302 neurons, eight of which produce dopamine (DA). Strains UA44 [*baInl1*($P_{dat\text{-}1}$::α-syn, $P_{dat\text{-}1}$::GFP)] and BY250 were used for DA neurodegeneration analyses. The six DA neurons within the anterior-most region of the animal were selectively assayed because differential sensitivity to toxins has been observed among worm DA neurons [17]. An adult worm was scored as normal when all six anterior neurons were intact. A worm was counted as degenerative when at least one of the six neurons showed degenerative phenotype such as cell body rounding, blebbing or neuronal process loss.

Each *sod-3*::GFP animal was imaged in the same region (anterior bulb of the pharynx) at the same magnification and exposure intensity [18]. Similarly, for the *hsp-4*::GFP transcriptional fusion reporter, the area scored was the intestinal region proximal to the pharynx. In both the assays, pixel intensity was quantified within a 100 × 100 μm box drawn in the same anatomical region and data were compiled across three replicates ($n = 30 \times 3$). Worm strains expressing either human α-syn (UA49, [*baInl2*; {$P_{unc\text{-}54}$::α-syn::GFP, *rol-6*(*su1006*)}]) or polyQ40 (AM141) under the control of bodywall muscle promoter, *unc-54*, were scored for aggregates as previously described [19,20]. PolyQ and α-syn aggregation analyses were performed in triplicate with L3 stage worms (30/trial). Inclusions of α-syn were scored on a scale where each value represented: no inclusions (0), few (1), moderate (2), or many (3); all polyQ aggregates were counted in the worm populations.

GFP was visualized in transgenic worms by immobilizing animals with 3 mM levamisole and mounting them onto 2% agarose pads. GFP fluorescence was examined with a Nikon Eclipse E800 epifluorescence microscope equipped with an Endow GFP HYQ filter cube (Chroma Technology). Images were captured with a Cool Snap HQ CCD camera (Photometrics) driven by MetaMorph Software (Molecular Devices).

Statistical analysis was performed using a Student's *t test* or one-way ANOVA followed by Tukey's post hoc test. Results were

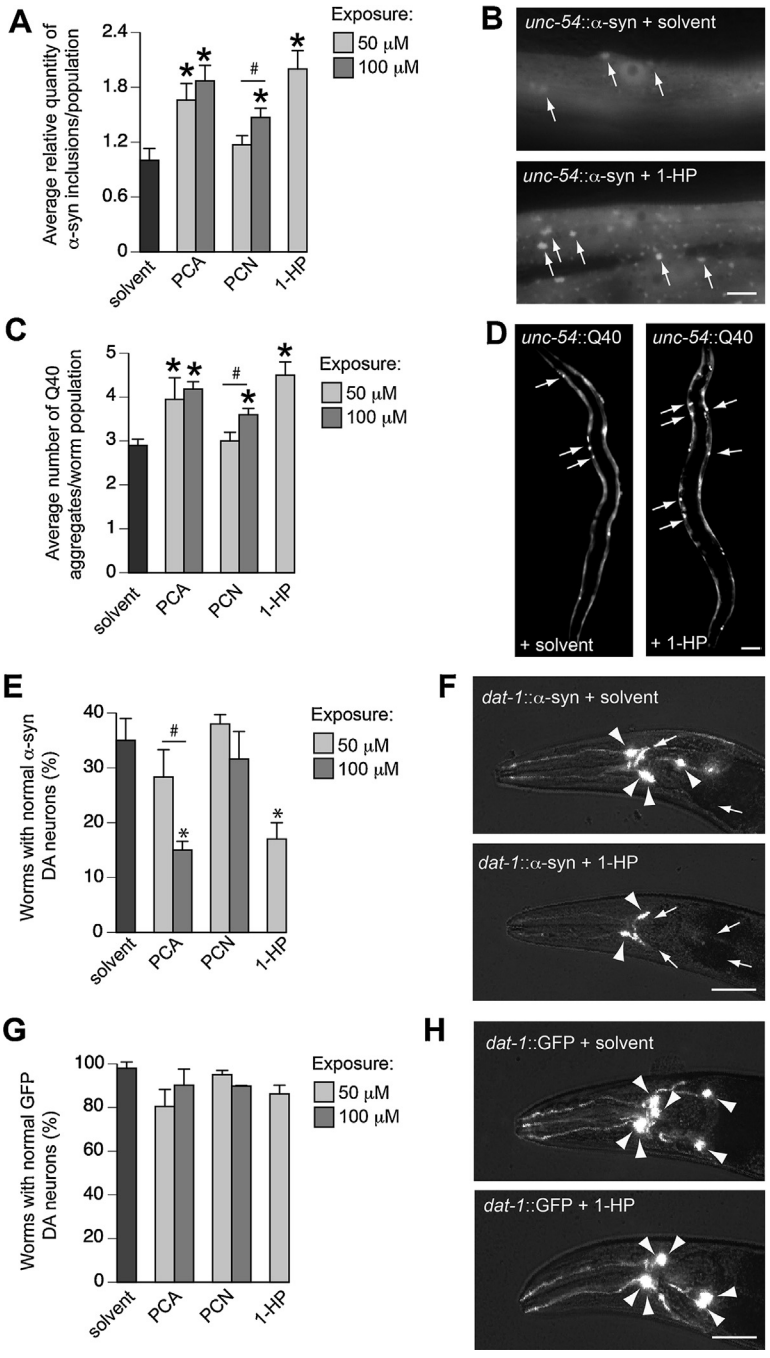

Fig. 2. Phenazine derivatives enhanced protein misfolding in bodywall muscles and caused hypersensitivity to α-syn-induced DA neurodegeneration in *C. elegans*. (A) Analysis of misfolded proteins within the worm population expressing α-syn::GFP showed a significant increase following phenazine treatments at day 3 compared to the solvent control. Inclusions of α-syn were scored on a scale of 0–3 where each value represents no inclusions (0), few (1), moderate (2), and many (3). These values are the mean ± SD of 3 experiments ($n = 30$/trial) ('*' represents significance between individual treatments and solvent control and '#' represents significance between two concentrations of the same phenazine derivative where $P < 0.05$ by one-way ANOVA). (B) Representative image of a worm (P_{unc-54}::α-syn::GFP) expressing the α-syn::GFP transgene in the bodywall muscle cells displaying misfolded protein (arrow) when treated with solvent control as indicated by the GFP puncta (top panel). When exposed to 1-HP the α-syn misfolding was enhanced (bottom panel). Scale bar, 5 μm. (C) Quantitative analysis of aggregate number within worm populations expressing Q40::YFP showed significant increases following phenazine treatments when compared to the solvent control at day 3. Values are the mean ± SD of 3 experiments where 30 animals were analyzed per replicate ('*' represents significance between individual treatments and solvent control and '#' represents significance between two concentrations of the same phenazine derivative where $P < 0.05$ by one-way ANOVA). (D) Representative images of worms expressing Q40::YFP in the bodywall muscle cells (P_{unc-54}::Q40::YFP) displaying few aggregated proteins (arrow) when exposed to solvent alone, as indicated by YFP puncta (left panel). When treated with 1-HP, polyQ aggregate number was enhanced (right panel). Scale bar, 50 μm. (E) Treatment with PCA and 1-HP enhanced α-syn-induced degeneration in DA neurons compared with solvent control alone at day 5. ('*' represents significance between individual treatments and solvent control where $P < 0.01$ by one-way ANOVA and '#' represents significance between the two concentrations of the same phenazine derivative where $P < 0.05$ by one-way ANOVA; $n = 30$ worms/3 trials). (F) Representative images of the experiment described in A. All *C. elegans* (P_{dat-1}::GFP + P_{dat-1}::α-syn) express GFP and α-syn specifically in the six anterior DA neurons. In all images, arrowheads show intact dopaminergic neuron cell bodies. Arrows indicate areas where dopaminergic neurons have degenerated. Scale bar, 50 μm. (G) Phenazine derivatives were examined in the absence of α-syn for independent effects on neurodegeneration at day 12 (P_{dat-1}::GFP only). None of these molecules caused significant degeneration compared to solvent control. Values are expressed as mean ± SD from 3 independent replicates. (H) Representative images of DA neurons following phenazine treatment in *C. elegans* in a background lacking α-syn (P_{dat-1}::GFP only), where the six anterior DA neurons remain intact, as indicated with arrowheads. Scale bar, 50 μm.

expressed as mean ± SD; differences with $P < 0.05$ were considered significant.

3. Results

Previous studies on phenazines and their association with oxidative stress and mitochondria led us to determine whether these phenazine derivatives increase mitochondrial-associated ROS production in vivo. In worms, this can be monitored using an oxidative stress reporter strain, *sod-3*::GFP, where GFP (green fluorescent protein) is under the control of the *sod-3* promoter. *sod-3* encodes a mitochondrial superoxide dismutase enzyme, which functions as an antioxidant [21]. Worms treated with PCA (100 μM), PCN (100 μM) and 1-HP (50 μM) exhibited significant upregulation of *sod-3*::GFP expression when compared to DMSO solvent control (Fig. 1A and B) ($P < 0.05$).

Various types of cellular stress, including oxidative stress, can overload ER function [22]. This causes an unfolded protein response or ER stress. In worms this response can be measured using a reporter strain *hsp-4*::GFP, which is homologous to mammalian ER chaperone, BiP, and is transcriptionally induced in response to ER stress [23]. In order to determine the effect of phenazines on ER stress response, worms were treated with the 3-phenazine compounds separately. PCA (50 μM and 100 μM) and 1-HP (50 μM) showed significant increase in ER stress response when compared to the solvent control (Fig. 1C and D). The level of *hsp-4* expression in PCN treated worms was similar to the control. This study is consistent with Vaccaro et al., where they reported that worms exposed to dibenzopyrazine/phenazine induced ER stress response [24].

Alterations in ER or mitochondrial homeostasis can be associated with the accumulation of misfolded proteins. Additionally, mitochondrial perturbations are a common theme in neurodegenerative proteinopathies such as PD and HD. These diseases are primarily caused by aggregation of proteins. In our model, we tested the sensitivity of the three phenazine molecules toward α-syn misfolding and polyglutamine aggregation in the bodywall muscles of *C. elegans*. α-Syn is a protein that is found to be misfolded in PD patients [25]. Hamamichi et al. showed that overexpression of wild-type human α-syn in bodywall muscles of *C. elegans* caused age-dependent α-syn misfolding in the cytoplasm, as visualized by GFP (P_{unc-54}::α-syn::GFP) [20]. We tested the impact of phenazine derivatives on α-syn misfolding in *C. elegans* and determined that all three phenazines exacerbated misfolding when compared to the solvent control (Fig. 2A and B). Moreover, PCA significantly increased α-syn misfolding at both 50 μM and 100 μM while PCN only did so at 100 μM. The remaining phenazine, 1-HP, significantly enhanced misfolding at 50 μM. Similarly, glutamine expansion (polyQ) leading to polyQ-dependent aggregation is an established cause of HD. We used a *C. elegans* polyQ model where the glutamine repeat number was at a threshold for misfolding [19] and stressors can push misfolding over the limit. These worms, expressing PolyQ40-YFP in the bodywall muscles (P_{unc-54}::Q40::YFP), exposed to PCA (50 μM and 100 μM), PCN (100 μM), and 1-HP (50 μM) significantly enhanced the polyQ aggregate numbers when compared to the solvent control (Fig. 2C and D). For both the α-syn and polyQ assays, both concentrations of PCA enhanced misfolding to a comparable level. In contrast, only the higher concentration of PCN (100 μM) caused misfolding in *C. elegans* expressing α-syn and polyQ; in both of these worm strains there was a significant difference between 50 μM and 100 μM exposure to PCN ($P < 0.05$) (Fig. 2A and C). These collective studies indicated that exposure to these phenazine molecules has a generalized effect in altering proteostasis.

We further investigated the effect of the three phenazines on protein misfolding, specifically in neurons, as accumulation of misfolded α-syn protein can lead to neurodegeneration. We previously established that co-expression of wild-type human α-syn and GFP in the DA neurons (P_{dat-1}::GFP; P_{dat-1}::α-syn) caused age- and dose-dependent neurodegeneration, as compared to DA neurons expressing GFP alone (P_{dat-1}::GFP) [20]. When exposed to PCA or 1-HP, α-syn expressing worms displayed significantly more DA neurodegeneration than non-treated control worms ($P < 0.05$) (Fig. 2E and F). To investigate a more general role for phenazines in DA neurotoxicity, these compounds were tested in a background lacking α-syn (GFP alone; P_{dat-1}::GFP), where animals do not normally display degenerative changes [20]. Following phenazine exposure, the DA neurons did not exhibit neurodegeneration (Fig. 2G and H).

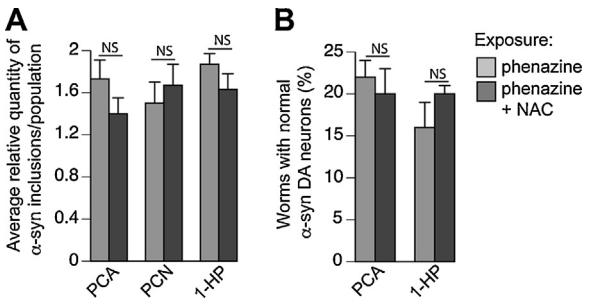

Fig. 3. Addition of an anti-oxidant failed to rescue toxic phenotypes caused by phenazine derivatives. (A) Treatment with 10 mM NAC (in DMSO), an anti-oxidant, could not rescue phenazine-induced α-syn misfolding in bodywall muscles as compared to phenazine treatments alone at day 3. The phenazine derivative concentrations examined were previously reported to induce an α-syn misfolding phenotype in *C. elegans* in Fig. 2A (100 μM PCA; 100 μM PCN; 50 μM 1-HP). (B) Similarly, NAC co-treatment also failed to attenuate the DA neurotoxicity caused by the phenazines in worms expressing α-syn at day 5. Phenazine derivative concentrations used were reported in Fig. 2E ($P > 0.05$; one-way ANOVA; $n = 3$ independent experiments).

In this study, we observed an increase in mitochondrial oxidative stress following exposure to phenazine molecules (Fig. 1A and B), consistent with previous findings [26,11]. To determine if excessive ROS production contributed to the neuronal cell death and protein misfolding phenotypes observed by phenazine treatment, we treated *C. elegans* with the anti-oxidant NAC (N-acetyl cysteine). Previous studies reported that NAC attenuated increased mitochondrial ROS caused by PCN and 1-HP [26,11]. We examined α-syn-induced DA neurodegeneration and α-syn protein misfolding in bodywall muscle cells of worms treated with phenazines or NAC and phenazines. These data indicate NAC (10 mM) does not rescue the phenazine-induced phenotypes previously observed (Fig. 3A and B). To provide support that NAC does reduce ROS production in *C. elegans*, *sod-3*::GFP worms were treated with 1-HP (50 μM) or 1-HP + 10 mM NAC. This antioxidant significantly reduced *sod-3*::GFP expression by 20% ($P < 0.05$; Student's *t*-test; data not shown). These findings indicate that cytotoxic effects induced by phenazines observed in *C. elegans* appear independent of oxidative stress.

4. Discussion

Mitochondrial defects and oxidative stress can impair protein homeostasis mechanisms, including the ubiquitin proteasome system (UPS) and the unfolded protein response (UPR). Bacterial metabolites such as DAPG (2,4-diacetylphloroglucinol) and a *Streptomyces venezuelae*-produced neurotoxin have been reported to impair mitochondrial function in model organisms [27,18]. Similarly, PCN impacts mitochondrial morphology of human epithelial cells and inhibits mitochondrial respiration by depleting cAMP and ATP levels [9]. Using *C. elegans* reporter strains, we showed that three phenazine derivatives caused an upregulation of *sod-3*

expression and increased UPR/ER stress response. Previously, PCN has been shown to be sensitive to sod1 and sod2 (cytosolic and mitochondrial superoxide dismutase) mutant strains in yeast, suggesting an involvement of mitochondrial ROS in its toxicity [28]. Oxidative modifications can disturb proteostasis, resulting in protein aggregation, as shown by the enhanced α-syn misfolding or PolyQ aggregation by phenazines in *C. elegans*.

Protein misfolding is a common pathological feature among neurodegenerative diseases. Under non-stressed conditions, neurons handle aberrant cellular proteins by various mechanisms, including recycling/degradation by the UPS and lysosomal degradation by autophagy. Under non-physiological conditions, such as oxidative stress or mitochondrial dysfunction, soluble proteins may oligomerize and form intracellular inclusions implicated in neuronal toxicity. We tested the capability of phenazine derivatives to cause neurodegeneration in the presence of α-syn as a triggering factor. Worms treated with phenazines were hypersensitive to α-syn-induced degeneration of DA neurons (Fig. 2E). We further showed that NAC, a radical scavenger, was incapable of attenuating the increased α-syn-induced aggregation and neurotoxicity caused by phenazines. This implies that oxidative stress may not play a causal role in phenazine-toxicity in our α-syn models. In a separate study using yeast cells, NAC was capable of partially rescuing the cytotoxic effects caused by PCN [28]. NAC can also rescue astrocytoma cells from increased mitochondrial ROS induced by PCN and 1-HP but fails to protect the concomitant cytotoxicity [11]. Moreover, mammalian cells treated with an autophagy inhibitor showed protection against PCN and 1-HP induced toxicity, thus indicating autophagy might mediate phenazine toxicity [11]. Further studies are necessary to investigate whether the enhanced DA neurodegeneration from phenazines in our α-syn model is impacted by autophagy.

A recent study in *C. elegans* revealed that dibenzopyrazine/phenazine reduced paralysis, neuronal toxicity, and oxidative stress in an ALS model with mutant TDP-43 [24]. It is possible that phenazine derivatives like PCA, PCN or 1-HP have diverse functional properties or bioactivity and may exhibit a different mode of action in *C. elegans*. It is therefore possible that the cellular conditions under which phenazine derivatives impart their activity is also distinct. Interestingly, Vaccaro et al. have also reported that dibenzopyrazine/phenazine causes increased ER stress response in worms, which is consistent with our results on the phenazine derivatives [24]. Due to the distinct physico-chemical properties of these phenazine compounds, an understanding of their individual and combined effects are required. Our data indicate that phenazine derivatives are capable of causing cellular stress, protein misfolding and neurotoxicity in *C. elegans*. These findings enlighten our understanding of mechanisms involving the cellular metabolism of phenazines as putative environmental sources of cytotoxicity contributing to neurodegenerative diseases.

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