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

Arpita Ray, Courtney Rentas, Guy A. Caldwell, Kim A. Caldwell*

Department of Biological Sciences, The University of Alabama, Tuscaloosa, AL 35487-0344, United States

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.

* Corresponding author. Tel.: +1 205 348 4021; fax: +1 205 348 1786.
E-mail address: kcaldwel@bama.ua.edu (K.A. Caldwell).

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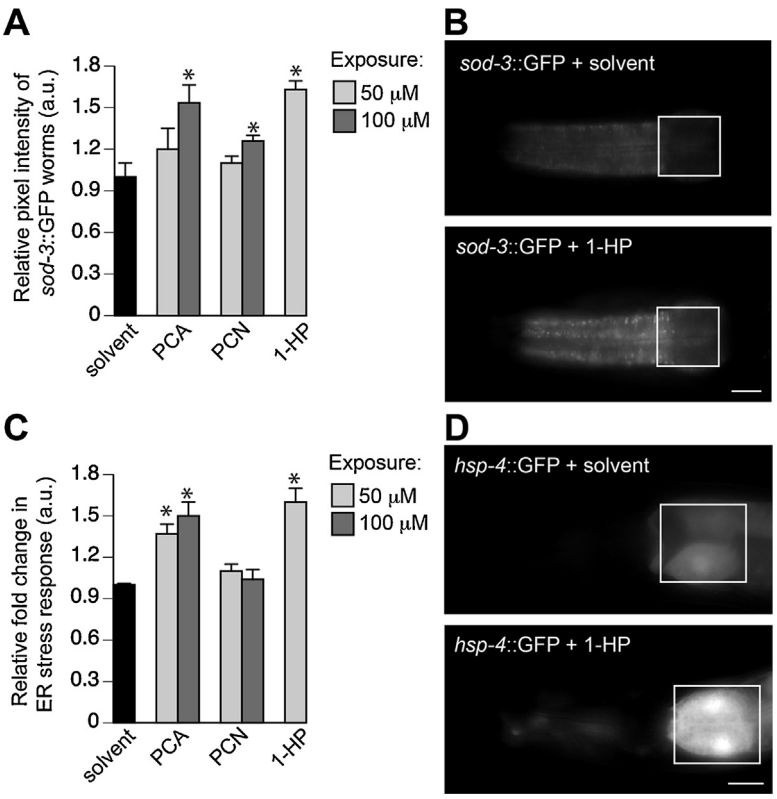

Fig. 1. Phenazine derivatives cause increased *sod*-3 expression and ER stress response in *C. elegans*. (A) Upregulation of *sod-3*::GFP expression, an indicator of oxidative stress, occurred following exposure to PCA, PCN, and 1-HP, compared with worms exposed to solvent only at day 4. This was quantitated 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