A bacterial metabolite induces glutathione-tractable proteostatic damage, proteasomal disturbances, and PINK1-dependent autophagy in *C. elegans*

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Gene-by-environment interactions are thought to underlie the majority of idiopathic cases of neurodegenerative disease. Recently, we reported that an environmental metabolite extracted from *Streptomyces venezuelae* increases ROS and damages mitochondria, leading to eventual neurodegeneration of *C. elegans* dopaminergic neurons. Here we link those data to idiopathic disease models that predict loss of protein handling as a component of disorder progression. We demonstrate that the bacterial metabolite leads to proteostatic disruption in multiple protein-misfolding models and has the potential to synergistically enhance the toxicity of aggregate-prone proteins. Genetically, this metabolite is epistatically regulated by loss-of-function to *pink-1*, the *C. elegans* PARK6 homolog responsible for mitochondrial maintenance and autophagy in other animal systems. In addition, the metabolite works through a genetic pathway analogous to loss-of-function in the ubiquitin proteasome system (UPS), which we find is also epistatically regulated by loss of PINK-1 homeostasis. To determine remitting counter agents, we investigated several established antioxidants and found that glutathione (GSH) can significantly protect against metabolite-induced proteostasis disruption. In addition, GSH protects against the toxicity of MG132 and can compensate for the combined loss of both *pink-1* and the E3 ligase *pdr-1*, a Parkin homolog. In assessing the impact of this metabolite on mitochondrial maintenance, we observe that it causes fragmentation of mitochondria that is attenuated by GSH and an initial surge in PINK1-dependent autophagy. These studies mechanistically advance our understanding of a putative environmental contributor to neurodegeneration and factors influencing in vivo neurotoxicity.

*Cell Death and Disease* (2015) 6, e1908; doi:10.1038/cddis.2015.270; published online 15 October 2015

Protein homeostasis (proteostasis) encompasses the process of translation, folding, compartmentalization, and degradation of proteins to maintain the long-term survival and functionality of the cell.¹⁻³ When proteins become misfolded they must be refolded or degraded to prevent disruptions to critical processes that result from proteotoxic stress.⁴⁻¹¹ Surveillance machinery that combats proteotoxic stress includes the ubiquitin proteasome system (UPS), retrograde chaperone-inducing signaling systems termed unfolded protein responses (UPR), and bulk destruction through autophagy. The cell also utilizes protein clearance machinery to induce the destruction of entire organelles, such as mitochondria, when they no longer function correctly.²⁻⁶ to protect the cell from reactive oxygen species (ROS). The last line of defense includes antioxidants in order to maintain a reduced intracellular state and attenuate damage to proteins.⁷⁻¹¹ Often, these regulated mechanisms are challenged by both the environment and genetic susceptibility factors. The integration of both, via gene-by-environment interactions, has been hypothesized to underlie many idiopathic neurodegenerative disorders.¹²⁻¹⁴ Understanding how the environment contributes to disease pathologies is important for understanding neurodegeneration.

Sources of environmental stressors are understudied and largely limited to human-derived toxicants such as pesticides like rotenone.¹⁴⁻¹⁵ However, people living in agricultural environs are often at a greater risk of developing neurodegenerative disorders that cannot be accounted for by human-derived toxicants alone.¹⁶ Environmental contributors may come from natural sources like metabolite-producing bacteria. For instance, bacterial sources have been reported to induce DOPA-responsive movement disorders in mice.¹⁷ Mechanistically, competition strategies among bacteria that produce antibiotics and small metabolites like phenazines that limit the growth of other bacterial species may have off-target effects on mitochondrial homeostasis, leading to ROS, protein damage, and neurodegeneration.¹⁸ Indeed, proteostatic dysfunction, altered mitochondrial dynamics, and elevated ROS production are characteristics of sporadic Parkinson’s disease (PD).¹⁹⁻²¹

Our laboratory previously demonstrated neurodegeneration induced by unreported small compounds within the growth media of the Gram-positive soil bacterium *Streptomyces venezuelae*.²²⁻²⁴ These bacterial products induce neuronal death in both *C. elegans* and cultured human neurons.²²

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Abbreviations: α-syn, α-synuclein; Aβ, amyloid beta; AA, ascorbic acid; BSO, butathionine sulfoximine; DCM, dichloromethane; EtAc, ethyl acetate; EV, empty vector; GFP, green fluorescent protein; GSH, glutathione; MT, melatonin; Pro, probucol; RNAi, RNA interference; ROS, reactive oxygen species; S. ven, *Streptomyces venezuelae*; UAc, uric acid; UPR, unfolded protein response; UPS, ubiquitin proteasome system; YFP, yellow fluorescent protein

Received 07.8.15; revised 14.8.15; accepted 17.8.15; Edited by E Baehrecke
disrupt mitochondrial complex I, induce ROS, and decrease ATP production. However, how these observations link to protein homeostasis has not been explored. Here we report that the active fraction of the S. venezuelae media induces disruptions in protein homeostasis, glutathione (GSH)-tractable α-synuclein toxicity, that UPS disruptions are epistatically regulated by loss-of-function to the PARK9 homolog pink-1, and that PINK1-dependent autophagy results in mitochondrial morphology disruptions. These observations indicate that pink-1 and UPS functionality are required for metabolite-induced protein toxicity in C. elegans, suggesting that these pathways may be linked and that environmental contributors to neurodegenerative disease may proceed through pathways implicated in familial forms.

Results

The S. venezuelae metabolite synergistically enhances toxicity associated with pathogenic protein expression in C. elegans neurons. S. venezuelae active fraction containing a small secondary product (MW <300) is isolated following growth of cells in liquid culture through extraction using dichloromethane (DCM) to separate compounds from the aqueous phase. The DCM fraction is evaporated completely and the solidified substance is resuspended in ethyl acetate (EtAc) as described; hereafter (for brevity and consistency) it will be referred to as the metabolite. EtAc is used as a negative solvent control in experiments and does not cause significant neurodegeneration.

PD is characterized by dopaminergic neuron loss and is associated with α-synuclein, which induces neurodegeneration when overexpressed or mutated. Consistent with this, α-synuclein overexpression in worm dopaminergic neurons using the dat-1 promoter (Pdat-1) induces neurodegeneration in the six anterior dopaminergic neurons (Supplementary Figure S1a). We performed a timecourse experiment in this genetic background and found that chronic metabolite supplementation (Supplementary Figure S1b) significantly decreased the percentage of animals with normal dopaminergic neurons at days 6–8 post hatching (Figures 1a and b). Animals at day 4 do not display enhanced neurodegeneration, suggesting that α-synuclein accumulation might not be sufficient for neurotoxicity manifestation. Animals expressing only GFP do not exhibit neurodegeneration at the time points and concentrations examined, potentially indicating a synergistic interaction between α-synuclein and the metabolite.

To determine whether these effects were limited to α-synuclein-induced neurodegeneration, we performed timecourse exposures in two other neurodegeneration models. First, human amyloid-β peptide (Aβ42), a toxic cleavage product of the amyloid precursor protein associated with Alzheimer’s disease, was examined in five glutamatergic tail neurons using the eat-4 promoter (Petat-4) (Supplementary Figure S1a). This expression induces neurodegeneration in a time-dependent manner and is modulated by factors, which promote proper proteostasis. Transgenic animals with Aβ42 expression had significantly decreased glutamatergic neuron counts at days 6 and day 10 when exposed to metabolite (Figures 1c and d). The metabolite had no effect on glutamatergic health in the absence of Aβ42.

Second, we examined metabolite effects on mutant huntingtin (Htn-Q150) expression under the control of the osm-10 promoter (Pom-10) in the C. elegans ASH-type sensory neuron. We assayed animals for defects in lipophilic dye uptake reported to be associated with Htn-Q150-induced disruption of ciliary endings. We discovered that dye-filling defects were significantly greater in metabolite-treated animals at days 6–10 (Figures 1e and f). Animals without pathogenic proteins do not display neurodegeneration. Taken together, these data suggest that the metabolite can enhance toxicity of neuronally-expressed pathogenic proteins in vivo.

The S. venezuelae metabolite is associated with proteostasis disruption. To determine whether neurodegeneration is correlated with alterations in protein handling, we monitored changes in apparent aggregate density or aggregate count of pathogenic proteins conjugated to fluorescent molecules in muscle cells with a semi-acute regimen of metabolite exposure (Supplementary Figure S1c) and potential behavioral alterations due to protein misfolding. First, we observed that the apparent aggregate density of α-synuclein was significantly increased upon metabolite treatment (Figures 2a and b). Second, animals with fluorescently-conjugated polyQ135 and polyQ40 had enhanced aggregate formation at two timepoints (Figure 2c; Supplementary Figures S2a and b). Furthermore, metabolite exposure to polyQ135 worms significantly impaired motility at the young adult stage (Figure 2d), potentially indicating behavioral alterations in response to aggregation. We did not observe metabolite-induced aggregate formation in subthreshold PolyQ19 or GFP only strains (data not shown), indicating that the metabolite alters the ability of threshold-state animals to handle polyQ misfolding. Finally, we extended studies to animals expressing Aβ42 in muscle cells under a temperature-sensitive promoter repression system and found that metabolite exposure induces enhanced paralysis at restrictive temperatures (23–25 °C) but not in animals that do not express Aβ42 (Figure 2e; Supplementary Figures S2c and d).

To exclude the possibility of transgenic expression artifacts, we monitored mutant phenotypes of animals bearing metastable protein alleles in RAS, let-60 (ga89), and paramyosin, unc-15 (e1401), which are highly sensitive to changes in the protein folding environment. We found that metabolite exposure in let-60 (ga89) animals but not in N2 animals significantly decreased brood viability (Figure 2f) and that motility (μm/s) was significantly reduced in unc-15 (e1402) but not in N2 animals (Supplementary Figure S2e). These observations indicate that broadly applicable proteostasis impairments are a consequence of metabolite exposure.

GSH attenuates metabolite-associated α-synuclein-induced proteotoxicity and proteosomal dysfunction. We previously demonstrated that the metabolite increases ROS in C. elegans lysates. To determine whether oxidative damage may be a component of protein mishandling we treated animals to antioxidants and then measured dopaminergic neurodegeneration or α-synuclein accumulation. Three antioxidants: ascorbic acid, uric acid, and probucol (Supplementary Figures S3a, c, and d) did not
Figure 1 The S. venezuelae metabolite synergistically enhances pathogenic protein toxicity in C. elegans neurons. Animals were treated chronically with 100 μl metabolite/EtAc (S. ven) solution [5 μl of the 1000 × concentrated metabolite/ml] or EtAc alone (solvent) on 35 mM plates as described in Supplementary Figure S1b. This solution is added to the grown OP50 bacterial lawn before animal transfer and the solvent is dried before use. (a) Animals expressing GFP alone or α-synuclein (α-syn) and GFP using the Pdat-1 promoter to target expression to dopaminergic neurons were assayed for altered neurodegeneration in response to S. ven at days 4, 6, 8, and 10 post hatching. (b) Animals without α-syn always display six anterior dopaminergic neurons (arrows), however neuron cell death is induced by α-syn expression (arrow heads) in a time- and metabolite-dependent manner. Scale bar, 10 μm. (c) Animals expressing GFP alone or the human Aβ42 peptide using the Pesf-4 promoter to target expression to glutamatergic neurons were assayed for altered neurodegeneration in response to S. ven at days 4, 6, 8, and 10 post hatching. (d) Animals without Aβ42 always display five posterior glutamatergic neurons (arrows), however neuron cell death is induced by Aβ42 expression (arrowheads) in a time- and S. ven-dependent manner. Scale bar, 10 μm. (e) Animals expressing GFP alone or mutant huntingtin (HtnQ150) and GFP using the Posm-10 promoter to drive expression to the ASH-sensory neuron were assayed for altered dye-filling behavior in response to S. ven at days 4, 6, 8, and 10 post hatching. (f) Animals without HtnQ150 display co-localization (arrow) in the ASH neuron of the endogenous GFP with the red DiI lipophilic dye (100 ng/μl), which inundates sensory neurons with exposed ciliary endings such as ASH, however loss of dye-filling (arrow head) as a result of neuron damage is induced by HtnQ150 in a time- and S. ven-dependent manner. Scale bar, 20 μm. Quantitative data in the above panels are represented as the mean ± S.D.; n = 40 animals per treatment per strain, replicated 3–4 times and analyzed using two-way ANOVA with Tukey’s post hoc test. *P < 0.05
Figure 2  The S. venezuelae metabolite induces proteostasis disruption. Observation of pathogenic protein aggregation is best studied in young animals (~ day 3 post hatching) as older animals tend to have saturated aggregation. Therefore, it was necessary to expose animals to a higher metabolite concentration in a dosing regimen referred to as ‘semi-acute’ (Supplementary Figure S1c) for examination of metabolite effects in this context. Animals for these assays were treated in a semi-acute manner by soaking L1-synchronized animals in metabolite solution [10–15 μl of the 1000 x concentrated metabolite/ml] or unextracted conditioned S. ven media (only for the Aβ paralysis experiment displayed in e) for 8 h. Solvent treatment for Aβ paralysis was SYZ media in artificial seawater used to grow the S. ven bacteria, otherwise solvent treatment refers to EtAc/buffer. Animals were assayed after semi-acute treatment at various time points for proteostasis disruption signified by loss of protein degradation and handling. (a) Animals expressing α-syn conjugated to GFP in the C. elegans body muscle cells under the control of the P unc-54 promoter were assayed 2.5 days (60 h) after treatment for apparent aggregate density using a qualitative 0–3 scale with the experimenter blind to the treatment condition being analyzed. Data represented as mean ± S.E.M.; n = 30 animals per treatment assessed in 3–4 replicates. **P < 0.01 Student’s t-test. (b) Representative C. elegans bodywall muscle cells expressing α-syn::GFP treated with solvent or S. ven metabolite. Scale bar, 5 μm. (c) Animals expressing a polyglutamine-35 tract (Q 35) conjugated to YFP within the C. elegans body muscle cells under the control of the P unc-54 promoter were treated with solvent or metabolite. They were assayed at time points before and after treatment corresponding to the L4 larval stage and 24 h after L4 by counting the number of aggregates present per animal. Data represented as mean ± S.E.M.; n = 30 animals per treatment assessed in 3–4 replicates. **P < 0.01. Data were assessed using Student’s t-test. (d) Animals bearing PolyQ35 in the bodywall muscle cells were exposed to the metabolite or solvent control and then examined with the MBF Bioscience Wormlab System for motility (μM/second) on a clean agar plate. n = 40–50 animals. Data represented as mean ± S.E.M were assessed using Student’s t-test. (e) Animals expressing Aβ42 peptide under the control of the bodywall muscle promoter (P myo-3) using a temperature-repression system (smg-1) were upshifted to 23 °C at the L3 larval stage to induce expression of Aβ; treatment is with S. ven metabolite or solvent. In parallel, animals that do not express Aβ were treated identically (overlapping gray lines) but did not exhibit paralysis. n = 90–120 animals per treatment, replicated 3–4 times. Data represented as mean ± S.E.M. were assessed using two-way ANOVA and Tukey’s post hoc test to assess for significance between each time point in the analysis to every other time point. *P < 0.05 for time points indicated. (f) let-60(ga89) brood fecundity was assessed per condition by counting the number of animals within the brood of a single animal that reached adulthood. n = 24 animal broods per replicate normalized to N2 solvent control. At least three replicates were utilized. Data represented as mean ± S.E.M (two-way ANOVA and Tukey’s post hoc test; **P < 0.01)
attenuated neurotoxicity whereas melatonin\(^{10}\) and GSH\(^{20}\) attenuated neurotoxicity (Supplementary Figure S3b, Figure 3a). Only GSH supplementation suppressed enhanced aggregate formation in \textit{C. elegans} body wall muscle cells (Figure 3b; Supplementary Figures S3e and f). GSH protects enzymes with open cysteine residues\(^{32}\) from oxidative damage, including enzymes of the UPS. In addition, we have previously demonstrated that proteasome impairments may occur from metabolite exposure.\(^{22}\) Therefore, to determine whether the metabolite induces proteotoxicity in part through UPS inhibition, we reduced proteasome function with a range of MG132 concentrations and identified that (at concentrations above 5 \(\mu\)M) MG132 induces neurodegeneration in the presence of \(\alpha\)-synuclein and that this neurotoxicity epistatically regulates metabolite-induced neurotoxicity (Supplementary Figure S4a). To corroborate these findings, we utilized an RNAi strain wherein the effects can be localized specifically to dopaminergic neurons expressing \(\alpha\)-synuclein\(^{13}\) and reduced the sole E1 activating enzyme \textit{uba-1} and six diverse 26S proteasome regulatory subunits (\textit{psmd-9, rpn-2, rpt-1, rpt-4, pas-3, and pbs-3}) in this strain. These gene knockdowns caused enhanced neurodegeneration (Figure 3c). Notably, metabolite activity is attenuated in these backgrounds, indicating that UPS loss-of-function epistatically regulates metabolite activity and suggests that UPS-linked protein homeostasis defects may result from metabolite exposure.

To link proteasomal dysfunction to GSH homeostasis, we treated animals bearing \(\alpha\)-synuclein with a combination of MG132, metabolite, and GSH and found that exogenous GSH was sufficient to protect against MG132 and/or metabolite toxicity (Figure 3d). In addition, GSH was observed to diminish MG132 and metabolite-induced heightened fluorescence of a proteasome-targeted fluorescence molecule, CFP::CL-1 (degron)\(^{22}\) expressed within \textit{C. elegans} dopaminergic neurons (Supplementary Figures S4b and c). Because metabolite toxicity was attenuated by exogenous GSH, we hypothesized that this toxicity may proceed through diminished GSH levels.\(^{34}\) To test this, we combined GSH homeostasis impairments with proteasomal disturbances and metabolite exposure in worm neurons expressing \(\alpha\)-synuclein. In both RNAi of the GSH synthesis gene \textit{gcs-1} in \textit{C. elegans} neurons and exposure to the GSH synthesis inhibitor buthionine sulfoximine (BSO)\(^{35}\) we found that combinations with MG132 and/or metabolite operate similarly in their neurodegeneration effect (Figures 3e and f). Furthermore, BSO can decrease proteasomal turnover of CFP::CL-1 in a manner similar to MG132 and metabolite (Supplementary Figure S4d). Therefore, it is possible that GSH homeostasis is a regulator of metabolite-induced proteotoxicity.

\textbf{Enhanced \(\alpha\)-synuclein toxicity is epistatically regulated by the PARK6 homolog, \textit{pink-1}.} Given that altered proteasome, protein mishandling, and GSH deficiencies correlate with mitochondrial dysfunction in sporadic PD,\(^{11,20,21,32,36}\) we sought to understand how metabolite-induced proteasome inhibition relates to PINK1 and Parkin. These two proteins associate with PD pathogenesis and regulate mitochondrial homeostasis, protein homeostasis, and autophagy in \textit{C. elegans} and other systems.\(^{6,37–41}\) First, to determine whether metabolite susceptibility depends on UPS-dependent Parkin, we depleted \textit{pdr-1} (the \textit{C. elegans} homolog) cell-autonomously in \(\alpha\)-synuclein-expressing dopaminergic neurons in conjunction with the metabolite and/or MG132. Knockdown of \textit{pdr-1} plus the addition of all three stressors produced a more severe degeneration phenotype than any two stressors alone (Figure 4a) whereas, with similar conditions, further enhancement of neurodegeneration was not observed in \textit{pink-1} (RNAi) animals (Figure 4b); here, these stressors appeared to act in a related manner. These data indicate that \textit{pink-1}, but not \textit{pdr-1}, may epistatically regulate proteasome inhibition and metabolite-induced protein toxicity.

Although qPCR data indicate that RNAi reduces gene expression by over 80\% in \textit{pdr-1} and \textit{pink-1} (RNAi) conditions (Supplementary Figure S5) seemingly parallel regulation of neurodegeneration observed in \textit{pdr-1} (RNAi), and UPS-metabolite toxicity conditions may be due to hypomorphic addition of stressors. Therefore, we crossed our \(\alpha\)-synuclein-expressing strain to \textit{pink-1} (tm1779), a null allele, and two alleles of \textit{pdr-1}: \textit{pdr-1} (tm598), an in-frame deletion\(^{40}\) and \textit{pdr-1} (gk448), a deletion predicted to remove the start codon for all predicted isoforms. The presence of \(\alpha\)-synuclein was largely required to parse genetic interactions, as few observable interactions were produced without \(\alpha\)-synuclein expression in these mutant backgrounds (Figure 4c).

Double mutants [\textit{pink-1} (tm1779); \textit{pdr-1} (tm598) or \textit{pink-1} (tm1779); \textit{pdr-1} (gk448)] demonstrate significantly greater (\(P<0.05\)) neurodegeneration than \textit{pdr-1} (tm598) or \textit{pdr-1} (gk448) alone (Figure 4d and e). When considering the interaction between loss of \(\alpha\)-synuclein and metabolite addition, these mutant lines confirm RNAi results, suggesting epistatic regulation of metabolite activity by loss of \textit{pink-1} (Figures 4d and e) but not \textit{pdr-1}, which appears to acts in parallel to metabolite toxicity.

At least two possibilities may account for these observations. One possibility is that \textit{pink-1} and \textit{pdr-1} operate independently to regulate \(\alpha\)-synuclein toxicity in worm dopaminergic neurons and loss of each gene is responsible for an additive phenotype observed in double mutants. Another possibility is that loss of one gene hypersensitizes worm DA neurons to loss of the other, meaning loss of both gene products results in a synergistically toxic state. Within this model, metabolite activity or loss of \textit{pink-1} may be specifically amplified by \textit{pdr-1} loss. To investigate these possibilities, we explored how GSH might protect against \textit{pink-1} and \textit{pdr-1} mutations (Figure 4f). We find that, although GSH cannot protect against \textit{pink-1} or \textit{pdr-1} loss-of-function in the context of \(\alpha\)-synuclein alone, the joint additive neurodegenerative phenotype of both \textit{pink-1} and \textit{pdr-1} was partially rescued by GSH (Figure 4f) to a degenerative state reminiscent of loss of \textit{pink-1} or \textit{pdr-1} alone. This evidence suggests that the enhanced degeneration observed from loss of \textit{pink-1} and \textit{pdr-1} gene products may not simply be additive but rather arise from a synergistic toxic mechanism that is attenuated by GSH supplementation.

\textbf{The metabolite induces mitochondrial morphological dysfunction and \textit{PINK1}-dependent autophagy.} GSH-tractable UPS inhibition is one mechanism by which the metabolite might exert a toxic influence on \(\alpha\)-synuclein-induced neurodegeneration. However, proteostasis also
involves efficient clearance of defective organellar protein compartments such as the mitochondria through autophagy. The link between UPS disturbances and autophagic induction remains unresolved, but at least one report has demonstrated that autophagy is induced as a compensatory mechanism for loss of UPS functionality. To test for altered autophagy, especially of mitochondria, a previously determined metabolite target, we utilized a combination of molecular and phenotypic assays.

One signifier for altered autophagic capabilities is represented by mitochondrial morphology, which is normally tubular (Figures 5a and b). We assessed this in response to metabolite exposure in animals bearing GFP fused to a mitochondrial import signal under the control of the muscle-specific myo-3 promoter. Compared with solvent treatment, populations of control (EV) RNAi animals treated with metabolite have significantly greater fragmentation characterized by circularly shaped mitochondria, which is potentially indicative of increased mitophagic activity due to decreased mitochondrial fission and more rapid turnover of mitochondria (Figures 5a and b). Only a few animals exhibited fused mitochondria (Figures 5a and b), which did not significantly change through metabolite exposure. When treated with GSH, metabolite-induced fragmentation was significantly reduced (Figures 5a and b), potentially linking GSH attenuation of mitophagy to mitochondrial morphology.

Next, we examined the regulation of mitochondrial morphology by PINK-1 and PDR-1. Compared with EV RNAi control populations, solvent-treated animals reduced for pink-1 and pdr-1 had significantly greater fragmentation (Figures 5a and b). Only a few animals exhibited fused mitochondria (Figures 5a and b), which did not significantly change through metabolite exposure. When treated with GSH, metabolite-induced fragmentation was significantly reduced (Figures 5a and b), potentially linking GSH attenuation of mitophagy to mitochondrial morphology.

Figure 3 GSH attenuates enhanced α-synuclein proteotoxicity and proteasomal dysfunction associated with the metabolite. Nemobates were exposed to the bacterial metabolite chronically for all neurodegeneration assays as described in the Figure 1 legend whereas animals were exposed to the metabolite semi-acute when they expressed alpha synuclein in bodywall muscle cells, as described in the Figure 2 legend. RNAi was performed in a worm strain whereby RNAi knockdown would occur only in dopaminergic neurons (cell-autonomous RNAi). (a) Animals expressing α-syn in the dopaminergic neurons were assessed for neurodegeneration in the context of 1 mM GSH. Data represented as mean ± S.D.; n = 30 animals analyzed per treatment in 3–4 replicates. ***P < 0.001 was assessed by two-way ANOVA with Tukey post hoc test. (b) Animals expressing α-syn in the bodywall muscle cells were assessed for apparent aggregate density in the context of 1 mM GSH. Data represented as mean ± S.D.; n = 30 animals analyzed per treatment in 3–4 replicates. ***P < 0.001 was assessed by two-way ANOVA with Tukey post hoc test. (c) Animals bearing dopaminergic overexpression of SID-1 (a d3RNA transporter) in a mutant background for α-syn, used to selectively target RNAi to dopaminergic neurons in an α-syn background were exposed to 1 mM IPTG plates and either empty vector control (EV) or RNAi treatment paradigms affecting the UPS at 6 days post hatching. RNAi was initiated at the L4 larval stage to exclude potential developmental defects. Data represented as mean ± S.D.; n = 30 animals analyzed per treatment in 3–4 replicates. ***P < 0.001 was assessed by two-way ANOVA with Tukey post hoc test. (d) Animals were treated with 10 μM MG132 with 1 mM GSH in the context of neurodegeneration through enhanced α-syn toxicity elicited by the S. ven. metabolite. EtAc and 0.1% DMSO were used where appropriate to serve as solvent controls. Animals were placed on MG132 concentrations at the larval L4 stage to exclude the possibility of developmental defects. Data represented as mean ± S.D.; n = 30 animals analyzed per treatment in 3–4 replicates. ***P < 0.001 was assessed by two-way ANOVA with Tukey’s post hoc test. (e) Animals were treated with 10 μM MG132 (using 0.1% DMSO as a solvent control) and metabolite as described in (a) in the context of cell-autonomous RNAi reduction of the GSH synthesis enzyme gcs-1 or empty vector control (EV). Data represented as mean ± S.D.; n = 30 animals analyzed per treatment in 3–4 replicates. ***P < 0.001 was assessed by two-way ANOVA with Tukey’s post hoc test. (f) Animals were treated with 10 μM MG132 as described in (a) in conjunction with 1 mM buthionine sulfoximine (BSO) in the context of neurodegeneration through enhanced α-syn toxicity elicited by the S. ven. metabolite. EtAc and 0.1% DMSO were used where appropriate to serve as solvent controls. Data represented as mean ± S.D.; n = 30 animals analyzed per treatment in 3–4 replicates. ***P < 0.001 was assessed by two-way ANOVA with Tukey’s post hoc test.

Discussion

Environmental contributors to neurodegenerative disease are less well studied than genetic potentiators. Nonetheless, characterization of environmental stressors offers the potential to enhance our understanding of idiopathic disease. Within bacteria, thousands of clades produce secondary metabolites, the majority of which are of unknown structure or activity and provide alluring targets for studies of unknown yet potentially ubiquitous stressors. Our work identified a potential

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microbial source for neurodegeneration represented in the *S. venezuelae* metabolite and implies that other bacteria may also produce neurodegeneration-inducing secondary metabolites. Our studies suggest that ROS\(^5\) and UPS perturbations act upon the innate toxicity of disease-linked pathogenic proteins by observing degeneration in *C. elegans* neurons and a deleterious increase of phenotypic readouts in bodywall muscle cells expressing misfolded proteins. The presence of pathogenic proteins can be important in disease progression through alterations of threshold states\(^31,52\) where homeostatic pathway dysregulation compounds over time. Consistent with this, no neurodegenerative phenotypes are observed in youthful animals in the absence of pathogenic protein expression, suggesting that the metabolite may act most strongly on threshold state animals. Mechanisms that link the UPS and ROS have yet to be fully explored, but our studies suggest that GSH regulation alters UPS activity and that this alteration is important for inducing toxic protein handling.
states. Furthermore, GSH appears to have a proactive role in compensating for UPS dysfunction, as GSH can protect against MG132 toxicity in the context of α-synuclein over-expression and dysregulation of GSH synthesis acts in a manner similar to MG132 treatment. GSH attenuation is hypothesized to occur through repairing damaged cysteine residues, which occur as a result of metabolite exposure; shifting the GSH couple to a more reduced state might beneficially alter the neurodegenerative threshold state of *C. elegans* cells.

Elucidating possible mechanisms for this metabolite might make use of pathways already defined for familial disease. It is a major hypothesis of disease pathogenesis that causative mutations within genes might be within very similar or even identical genetic pathways. Therefore, it is possible that UPS perturbations and ROS induction may collaborate with PINK1 and Parkin pathways in the context of Parkinsonism. In our studies, we defined a pathway comprises the UPS and GSH homeostasis that is epistatically regulated by loss of *pink-1*. The *C. elegans* Parkin homolog appears to act in parallel to PINK1 dysfunction, despite the importance of Parkin toward mitochondrial maintenance and in ameliorating α-synuclein stress. However, we hypothesize that due to the nature of *pdr-1* loss-of-function that seemingly parallel regulation of metabolite toxicity may actually be a synergistic toxic state where *pdr-1* loss-of-function interacts with *pink-1* loss-of-function in undefined, yet GSH-tractable, ways (as neurodegeneration in double mutants can be partially rescued by GSH).

Finally, we wished to provide a context by which *pink-1* loss-of-function may epistatically regulate toxicity associated with metabolite exposure by investigation of autophagic capabilities regulated by PINK-1. We discovered that the metabolite, as well as depletion of *pink-1* or *pdr-1*, induced mitochondrial fragmentation, which we hypothesized may be due to alterations in autophagic capabilities. However, although GSH attenuated fragmentation resulting from metabolite exposure, GSH did not attenuate metabolite-induced mitochondrial fragmentation in *pink-1(RNAi)* backgrounds. To explain this, we found that in *pink-1* (RNAi) backgrounds, LGG-1 recruitment and upregulation (which are induced in metabolite-treated animals) is impaired. This is consistent with evidence that decreased PINK1 signaling impairs stress-induced autophagy.

These data as a whole indicate that the metabolite can induce proteostatic deficiencies through GSH-tractable UPS impairments, which may increase the need for bulk autophagy. This state produces fragmented mitochondria potentially as an indicator of mitochondria undergoing autophagy. The metabolite also elicits neurodegeneration in animals bearing pathogenic proteins and accumulation of damaged proteins in worm bodywall muscle cells. Reduction of PINK-1 signaling disrupts this paradigm and animals become intractable to GSH attenuation. We hypothesize that this reduction supercedes metabolite-induced neurotoxicity, potentially due to the nature of PINK-1 loss-of-function, loss of autophagy induction, and accumulation of mitochondrial fragmentation. In the future, research defining relationships among *PINK-1*, UPS, and autophagy in neurons may help to further elucidate the connection between these pathways and neurodegeneration. From a gene-by-environment perspective, it should be noted that three aspects of idiopathic PD: proteasome function deficiencies, depleted GSH intracellular concentrations, and mitochondrial dysfunction, mechanistically intersect within the literature and also when *S. venezuelae* metabolite disrupts cellular processes. Therefore, environmental stress may proceed via GSH-tractable intracellular deficiencies, which connect seemingly diverse homeostatic pathways.

**Materials and Methods**

*C. elegans* strains. *C. elegans* were grown and maintained using standard procedures. The following strains were provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440): N2 (Bristol), DA1240 (adt-5[124pdat-1::GFP]), H3 (lin-15[tm185]/nus-1[tm125::GFP]), HT2 (lin-15[2]; [Pdat-1::GFP], n36[::GFP]), and HT4 (lin-15[2]; [Pdat-1::GFP], n36[::GFP]). An environmental cause of proteostatic dysfunction

![Figure 4](image-url) Enhanced α-synuclein toxicity is epistatically regulated by the PAR6 homolog, *pink-1*. Neurodegeneration was assessed on animals treated chronically with the bacterial metabolite as described in Figure 1a. RNAi was performed in a strain whereby RNAi knockdown would occur only in dopaminergic neurons (cell-autonomous RNAi). Animals were reduced through cell-autonomous RNAi for *pdr-1* (RNAi) or *pink-1* (RNAi) in conjunction with the *S. venezuelae* and/or 10 μM MG132. Animals were treated until day 6 post hatching. Solvent controls include EAC or 0.1% DMSO. Data represented as mean ± S.D.; n = 30 animals per treatment analyzed in 3–4 replicates. **P < 0.0001** was assessed by two-way ANOVA with Tyuwe’s post hoc test. (c) Animals with a GFP-only expression construct in dopaminergic neurons were crossed to alleles for *pink-1* (tm1779) and *pdr-1* (tm598) mutant lines. These animals were treated with the metabolite until day 12 post hatching. Animals were analyzed per treatment in 3–4 replicates. **P < 0.05** was assessed using two-way ANOVA with Sidak’s post hoc test. An environmental cause of proteostatic dysfunction

![Figure 4](image-url) Enhanced α-synuclein toxicity is epistatically regulated by the PAR6 homolog, *pink-1*. Neurodegeneration was assessed on animals treated chronically with the bacterial metabolite as described in Figure 1a. RNAi was performed in a strain whereby RNAi knockdown would occur only in dopaminergic neurons (cell-autonomous RNAi). Animals were reduced through cell-autonomous RNAi for *pdr-1* (RNAi) or *pink-1* (RNAi) in conjunction with the *S. venezuelae* and/or 10 μM MG132. Animals were treated until day 6 post hatching. Solvent controls include EAC or 0.1% DMSO. Data represented as mean ± S.D.; n = 30 animals per treatment analyzed in 3–4 replicates. **P < 0.0001** was assessed by two-way ANOVA with Tyuwe’s post hoc test. (c) Animals with a GFP-only expression construct in dopaminergic neurons were crossed to alleles for *pink-1* (tm1779) and *pdr-1* (tm598) mutant lines. These animals were treated with the metabolite until day 12 post hatching. Animals were analyzed per treatment in 3–4 replicates. **P < 0.05** was assessed using two-way ANOVA with Sidak’s post hoc test. A separate statistical test using two-way ANOVA with Tyuwe’s post hoc test (not shown here) demonstrates a statistical difference between solvent-treated *pdr-1* and *pdr-1;pink-1* double-mutation, suggesting an additive phenotype. (e) Animals with the *pink-1* (tm1779) mutation and/or the *pdr-1* (gk448) deletion were crossed into nematodes expressing both the GFP and α-syn transgenes in the dopaminergic neurons. Homozygosity was confirmed by PCR. These animals were treated until day 6 post hatching. Data represented as mean ± S.D.; n = 30 animals analyzed per treatment in 3–4 replicates. **P < 0.05** was assessed using two-way ANOVA with Sidak’s post hoc test. A separate statistical test using two-way ANOVA with Tyuwe’s post hoc test (not shown here) demonstrates a statistical difference between solvent-treated *pdr-1* and *pdr-1;pink-1* double-mutation, suggesting an additive phenotype. (f) Strains treated in (d) were treated with 1 mM GSH. Data are represented as mean ± S.D.; n = 30 animals analyzed per treatment in 3–4 replicates. **P < 0.05** was assessed using two-way ANOVA with Sidak’s post hoc test.
PAut-1::GFP], UA271 (pink-1(tm1779); pdr-1(tm598); baln1[Pdat-1::α-synuclein, Pdat-1::GFP]), UA279 (pdr-1(gk448); baln1[Pdat-1::α-synuclein, Pdat-1::GFP]), and UA280 (pink-1(tm1779); pdr-1(gk338); baln1[Pdat-1::α-synuclein, Pdat-1::GFP]).

**Construction of pink-1(tm1779), pdr-1 (tm598), and pdr-1(gk448) containing strains.** When crossing mutant strains containing these alleles, the molecular lesions are easily probed using the following primers:

- **pink-1(tm1779)** Forward: GTT ACAAGGCGAGCCTGAAAG
  Reverse: GAAGCCTCGGGCTT ATT AAGG

- **pdr-1(tm598)** Forward: CAGACAAA TCA TGCTTCTCCG
  Reverse: CGTCTTCGCTCTGGCACAC

- **pdr-1(gk448)** Forward: CACTT ACGCAAGTGCTTCTTCG
  Reverse: GT ACGTGAGTT AGAGCTGC

In all cases the mutant animals were crossed into strain UA44 (baln1[Pdat-1::α-synuclein, Pdat-1::GFP]) to generate strains UA86, UA88, UA271, UA279, and UA280; and into BY200 (vtls1[Pdat-1::GFP, rol-6(su1006)]) to generate UA226 and UA227.

**Isolation and extraction of *S. venezuelae* metabolite.** Metabolite was generated as previously described. Briefly, spores from the *S. venezuelae* strain (ARS NRRL ISP-5230) were inoculated in 5 liters of SYZ media in artificial seawater and grown at 30 °C in a shaker. Samples were harvested after 3 weeks. Cell debris was removed by centrifugation at 10,000 × g for 10 min and supernatants were sequentially passed through 4–6 PES filter membranes with the following range of pore sizes: 11, 6, 2.7, 1.2, 0.7, 0.45, and 0.22 μM. The resulting conditioned media was extracted with an equal volume of DCM using a separatory funnel (2–3 times). The DCM layer was collected, dried, and the residue was resuspended in EtAc to a 1000-fold concentrated stock solution compared with the original volume of conditioned media. We calculate working concentrations of metabolite of this concentrated stock solution. Therefore, a 1 × concentration is 1 μl
Figure 5  The S. venezuelae metabolite elicits GSH-tractable mitochondrial fragmentation and induces PINK-1-dependent autophagy. Nematodes were exposed to the bacterial metabolite chronically for all assays as described in the Figure 1 legend. RNAi was performed systemically using target genes as indicated in various experiments. (a) Animals expressing a mitochondrial-targeted GFP signal expressed in the C. elegans bodywall muscle cells were morphologically assessed during different combinations of S. ven, GSH, and RNAi treatment. Images depict representative phenotypes for each condition tested. The shaded boxes indicate the morphology represented in each [fused (also shown with an arrow), fragmented, or normal], as described in (b). Scale bar, 20 μm. (b) The distribution of mitochondrial morphology, characterized as either normal (the majority of mitochondria are tubular), fragmented (the majority of mitochondria are circular or irregularly shaped), or fused (elongated or convoluted structures) for each condition tested is shown as a percentage in the population. The white-dashed line indicates the threshold for normal mitochondrial morphology in EV controls treated with solvent, EtAc. n = 30 animals analyzed per treatment in 3–4 replicates. *P < 0.05 was assessed by two-way ANOVA with Dunnet’s post hoc test to compare different treatment groups with EtAc control within each RNAi treatment group. A separate (but not shown) statistical test using two-way ANOVA and Sidak’s post hoc test reveals a statistical difference between solvent-treated pink-1(RNAi) and pdr-1(RNAi) when compared with the EV control in the context of mitochondrial fragmentation (c and d). Animals expressing an N-terminally fused mCherry::LGG-1 (LC3) construct were assessed for increased stress granule formation in the context of the S. ven metabolite and pink-1(RNAi); lgg-1 and bec-1 RNAi were used as negative controls. mCherry::LGG-1 puncta were counted in each of three rectangular boxes of 200 × 200 μm; they were placed in tandem beginning with the most posterior region of the intestinal and extended toward the vulva. Arrows indicate representative puncta. n = 30 animals analyzed per treatment in 3–4 replicates. ****P < 0.0001; one-way ANOVA followed by a Tukey’s post hoc test. Data represented as mean ± S.D. Scale bar, 10 μm. (e) Animals exposed to empty vector (EV) or pink-1(RNAi) were assessed for lgg-1 mRNA levels with or without S. ven metabolite treatment. This treatment occurred until the L4 stage. Three replicates comprises at least 100 animals each; 1 μg of RNA was subjected to cDNA synthesis. Primers used for RT-qPCR are listed in Materials and methods. At least three stable reference genes were used. Data represented as mean ± S.E.M. **P < 0.01 and ****P < 0.0001 were assessed using Q-base software.
of metabolite resuspended in 1 ml EtAc. In our assays, chronic exposure utilizes a 5 x concentration for neurodegeneration assays and 20 x concentration for autophagy and mitochondrial morphology, while semi-acute soaking associated with pathogenic protein aggregation utilizes 10–20 x concentrations.

**Chronic metabolite treatment.** All drugs were provided at final concentrations as indicated in figures. Animals were either exposed to metabolite chronically or in a semi-acute manner (Supplementary Figure S1). The purpose of the chronic treatment paradigm, described here, was to provide consistent long-term exposure to the metabolite and was used for neurodegeneration associated with N2 (Bristol), UA96 (unc-119(e363), bali19 [Pαf-1::CFP::CL-1], unc-119(e363)), BY250 (vts[Pαf-1::CFP::GFP]), UA44 (baln1[Pmyo-3::cytochrome, P αf-1::CFP::GFP]), UA196 (sid-1(pk3231f), baln33Pαf-1::sid-1, Pαf-1::mCherry), DA1240 (adls124[Pαf-1::CFP::GFP]), UA198 (baln34Pαf-1::Aβ–20, Pmyo-3::mCherry), HA93 (lin-15(b18;765), nus11[Pαf-1::CFP::GFP lin-15(b18;765)]), and HA659 (dry-20(e2182); vts1[Pαf-1::CFP::GFP, Pαf-1::HnQ150, dry-20(e2182)]) as well as autophagy associated with the strain VC1093 [Pmyo-3::mCherry; LGG-1] and mitochondrial morphology associated with the strain SJ4103 (acvs1[Pmyo-1::CFP::GFP]).

Metabolite solution [0.5 μl (5 x) or 2 μl (20 x) of the concentrated stock solution is reconstituted in 100 μl EA or EtAc (solvent control) alone was placed within the bacterial lawn and allowed to dry. After, mixed staged animals are placed on each treatment plate and allowed to reach gravid adulthood. These animals are used for egg laying. This is a pretreatment paradigm, which we have previously shown produces a stronger and more consistent result.25 Two days after hitting 100 μl 4 ml agar of 5 × metabolite in the M9 salt buffer (3 mg/ml KH2PO4, 6 mg/ml Na2HPO4, 12.5 μg/ml ampicillin) for 16 h at 37 °C. RNAI bacteria is then placed onto NFG IPTG-ampicillin analysis plates (NGM: 1 mM IPTG, 100 μg/ml ampicillin) at 250 μl/4 ml NGM for 24 h at 22 °C. The RNAI clones pink1([JED86-9], bai-1(C47E12.5), pmd-9;[JCB47.1], r22;[C260I14.4], rpi-1([C52E4.1], rdt-4([P23F1.8], pass-3(Y1104A14.1), pbs-2(Y38A8.3), gck-1(C32G25.1), bcl-1([T19E7.3]), and L4440 (EV) were obtained from the Ahringer Library.55, pdr-1([KOE83.7]) was generated in our laboratory.27 In most cases, nematodes were exposed to RNAI bacteria for two generations. Proteasome RNAI analysis was postponed until the the L4 molt (~60 h post hatching) to exclude developmental impairments.

**Drug treatments.** Dil (Biotum, Hayward, CA, USA) was used at a final concentration of 100 ng/μl. MG132 (Cayman Chemical, Ann Arbor, MI, USA) was used at a concentration of 1–50 μM (see Supplementary Figure S3A) in DMSO. BSO (Enzo Life Sciences, Ann Arbor, MI, USA) was utilized at a 1–mM concentration dissolved in water. Antioxidants were brought to a final concentration of 1 mM in NGM plates by dissolving in the media during preparation. These drugs were ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA), uric acid (Alfa Aesar, Ward Hill, MA, USA), probucol (MP Biomedicals, Santa Ana, CA, USA), melatonin (Sigma-Aldrich), and GSH (Sigma-Aldrich). Probucol was dissolved in ethanol, the final volume of which did not exceed 1%. All GSH experiments were performed using drug plates with 1 mM concentration throughout the entirety of the experiment refreshed every 2 days.

**Analysis of dopaminergic neurodegeneration.** This assay was performed on animals containing α-synuclein in dopaminergic neurons (UA44 (baln1[Pαf-1::cytochrome, P αf-1::CFP::GFP]), UA196 (sid-1(pk3231f), baln33Pαf-1::sid-1, Pmyo-3::mCherry); baln1[Pαf-1::CFP::GFP, P αf-1::CFP::GFP], UA86 (pdr-1(m598); baln1[Pαf-1::cytochrome, P αf-1::CFP::GFP]), and UA271 (pdr-1(m598); baln1[Pαf-1::cytochrome, P αf-1::CFP::GFP] or GFP alone animals BY250 (vts[Pαf-1::CFP::GFP], BY200 (vts[Pαf-1::CFP::GFP, rol-6(su1006)], UA226 (pdr-1(m598); vts[Pαf-1::CFP::GFP, rol-6(su1006)], UA227 (pdr-1(gk449); baln1[Pαf-1::cytochrome, P αf-1::CFP::GFP], and UA280 (pdr-1[m598]; pdr-1(pk3231f); baln1[Pαf-1::cytochrome, P αf-1::CFP::GFP]). In all, 30–40 animals were assessed for the presence of six anterior soma as well as their associated processes. Animals with missing soma or missing processes are considered as degenerating. Data were assessed by two-way ANOVA using Tukey’s post hoc test.

**Analysis of glutamatergic neurodegeneration.** Animals containing the Aβ42 cleavage product expressed in glutamatergic neurons UA198 (baln34Pαf-1::Aβ–20, Pmyo-3::mCherry)), adls124(Pαf-1::GFPII) or GFP alone DA1240 (adls124[Pαf-1::GFPII]). In all, 30–40 animals are assayed for the presence of five glutamatergic neurons in the tail as well as their associated canonical processes. Data were assessed by two-way ANOVA using Tukey’s post hoc test.

**Analysis of lipophilic dye uptake.** Huntingtin-induced neurodegeneration in the ASH-sensory neuron using the strains that contain the Huntingtin-Q50 repeat HA659 (dry-20(e2182); vts1[Pαf-1::CFP::GFP, Pαf-1::HnQ150, dry-20(e2182)]) or GFP alone HA 3 (lin-15(b18;765), nus11[Pαf-1::CFP::GFP, lin-15(b18;765)])) were assayed for colocalization between endogenous GFP in the ASH-sensory neuron with the red Di lipophilic dye by soaking animals on the day of analysis for 1 h in 1% DIOH, 100 ng/μl Dil in M9 buffer at room temperature. In all, 30–40 animals were analyzed. Data were assessed by two-way ANOVA using Tukey’s post hoc test.

**Analysis of α-synuclein aggregation.** Thirty animals containing body wall α-synuclein UA49 (baln34Pαf-1::cytochrome, P αf-1::CFP::GFP, rol-6(su1006))) were assayed 60 h after semi-acute exposure to the metabolite. Aggregate numbers were qualitatively determined on a 0–3 scale (0 none, 3 many) in worm lines26 wherein the experimenter was blinded to the treatment condition. Data were assessed by Student’s t-test.

**Analysis of PolyQ aggregation.** Thirty animals containing PolyQ repeat length polymorphisms, AM141 (m3132[Punc-32::Q35::YFP]) and AM141 (m3133[Punc-32::Q5::YFP]).
Average motility (100 frames using the MBF Bioscience Wormlab System (Williston, VT, USA). Animals were placed in the center of a scoring plate and measured for a minimum of 30 min. Approximately 10 clean animals were placed in the center of a scoring plate to clear animals of bacteria for approximately 10 min. Approximately 10 clean animals were placed in the center of a scoring plate until analysis at the young adult stage (generally 24 h after L4 molt). At the young adult stage we performed a semi-acute exposure. Worms were exposed for two generations of RNAi treatment (EV, pdr-1, and lgg-1) transgenic animals were analyzed at day 4 post hatching following 20 μM metabolite treatment. To count puncta of mCherry::LGG-1, three 200 × 200 pixel regions of interest were selected and thirty puncta were measured from at least 20 cells per treatment group. A 70-pixel diameter circle (chronic metabolite and/or pharmacological treatment) at day 7 post hatching. One PDE neuron from each animal was imaged using standardized magnification and exposure values across all animals in all treatment groups. A 70-pixel diameter circle (approximately 0.1 μm/pixel) was placed such that it encompasses the nucleus and the majority of the cytosolic compartment of the neuron. Average fluorescence values across all pixels within the circle were measured. Pools of 20 nuclei per replicate were averaged. Statistical tests were performed using a two-way ANOVA with the mean and standard error of the mean of each treatment group.

Fluorescence microscopy. Worms were immobilized with 3 mM levamisole and mounted on 2% agarose pads on a microscopic slide. Fluorescent microscopy was performed using a Nikon Eclipse E800 epifluorescence microscope equipped with an Endow GFP HYQ filter cube (Chroma Technology, Bellows Falls, VT, USA) or a Texas Red filter cube (Chroma Technology). A Cool Snap CCD camera (Photometrics, Tuscon, AZ, USA) driven by the MetaMorph software (Molecular Devices, Sunnyvale, CA, USA) was used to acquire images.

Analysis of metastable protein allele unc-15(e1402) and polyQ35 motility. Mixed populations of N2 worms and unc-15(e1402) animals or polyQ35 were maintained at 16 °C. Populations were synchronized by hypochlorite treatment. Twenty-four hours later animals were semi-acutely exposed to the metabolite solution in M9 buffer at the L1 stage for 8 h at 16 °C then placed upon a recovery plate until analysis at the young adult stage. At the young adult stage we performed a semi-acute exposure. Worms were exposed for two generations of RNAi treatment (EV, pink-1 or pdr-1) and maintained at 16 °C throughout the entire experiment. At the young adult stage, we then placed a solitary worm in a 24-well plate for 24 h to lay eggs. After 24 h, the adult worm was cleared from the plate. Plates were then supplemented every 24 h with fresh metabolite solution. Ninety-six hours later, the number of animals per brood that had attained adulthood were counted. Each replicate contains 24 worm broods that were averaged. Each treatment group was normalized to the N2 solvent control. All three replicates were used. Statistical tests were performed using a two-way ANOVA with a Tukey multiple comparison test (GraphPad Prism, La Jolla, CA, USA).

Autophagy analysis. For autophagy analysis, 30 V1903 (P_{pdr-1:mCherry}: LGG-1) transgenic animals were analyzed at day 4 post hatching following 20 μM metabolite treatment. To count puncta of mCherry::LGG-1, three 200 × 200 μm boxes were assigned from posterior to anterior along the intestine. The first box was placed at region at the most posterior portion of the intestine. The second was placed anterior to this position, extending toward the vulva, etc. Data were analyzed by one-way ANOVA and Tukey’s post hoc test.

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Mitochondrial morphology analysis. SJ4103 (zcds14(P_{pmp-3}:GFP::MYC)) animals were exposed for two generations of RNAi treatment (EV, pink-1 or pdr-1) in the presence of the metabolite or solvent control. Animals were treated (Supplementary Figure S1B) for 7 days post hatching using a 20 μM concentration of metabolite by chronic supplementation to the worm media. For GSH treatment, animals were placed on plates with 1 mM GSH dissolved within the media for the entirety of the analysis. Three muscle cells were analyzed per worm and thirty worms were analyzed per experiment. Data were analyzed by two-way ANOVA and Dunnett’s and Sidak’s post hoc test. Normal cells have ordered, ribbon-shaped mitochondria with few circular forms. Fragmented cells consist of disorganized circular forms and fused mitochondria have many ribbon-shaped mitochondria connected in a long labyrinthine formation.

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