Mitochondrial dysfunction, oxidative stress, and neurodegeneration elicited by a bacterial metabolite in a C. elegans Parkinson’s model

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Genetic and idiopathic forms of Parkinson’s disease (PD) are characterized by loss of dopamine (DA) neurons and typically the formation of protein inclusions containing the alpha-synuclein (α-syn) protein. Environmental contributors to PD remain largely unresolved but toxins, such as paraquat or rotenone, represent well-studied enhancers of susceptibility. Previously, we reported that a bacterial metabolite produced by Streptomyces venezuelae caused age- and dose-dependent DA neurodegeneration in Caenorhabditis elegans and human SH-SY5Y neurons. We hypothesized that this metabolite from a common soil bacterium could enhance neurodegeneration in combination with PD susceptibility gene mutations or toxicants. Here, we report that exposure to the metabolite in C. elegans DA neurons expressing human α-syn or LRRK2 G2019S exacerbates neurodegeneration. Using the PD toxin models 6-hydroxydopamine and rotenone, we demonstrate that exposure to more than one environmental risk factor has an additive effect in eliciting DA neurodegeneration. Evidence suggests that PD-related toxicants cause mitochondrial dysfunction, thus we examined the impact of the metabolite on mitochondrial activity and oxidative stress. An ex vivo assay of C. elegans extracts revealed that this metabolite causes excessive production of reactive oxygen species. Likewise, enhanced expression of a superoxide dismutase reporter was observed in vivo. The anti-oxidant probucol fully rescued metabolite-induced DA neurodegeneration, as well. Interestingly, the stress-responsive FOXO transcription factor DAF-16 was activated following exposure to the metabolite. Through further mechanistic analysis, we discerned the mitochondrial defects associated with metabolite exposure included adenosine triphosphate impairment and upregulation of the mitochondrial unfolded protein response. Metabolite-induced toxicity in DA neurons was rescued by complex I activators. RNA interference (RNAi) knockdown of mitochondrial complex I subunits resulted in rescue of metabolite-induced toxicity in DA neurons. Taken together, our characterization of cellular responses to the S. venezuelae metabolite indicates that this putative environmental trigger of neurotoxicity may cause cell death, in part, through mitochondrial dysfunction and oxidative stress.

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Parkinson’s disease (PD) is associated with dopaminergic neurodegeneration. Pathologically, this disease involves accumulation of the alpha-synuclein (α-syn) protein within proteineous inclusions called Lewy Bodies.¹ Current neurodegeneration research is focused on identification of the causative factors and underlying mechanisms. Around 10% of PD cases are caused by genetic factors. Unknown factors, including environmental exposures (heavy metals and agricultural chemicals such as paraquat and rotenone), are associated with most cases of parkinsonism.²⁻⁶ There is also a higher incidence of PD in rural areas where the odds ratio cannot be completely accounted for by the level of toxic exposures often encountered by the use of chemicals in farming.⁷ Associated with rural living, individuals exhibit a much greater interaction with the surrounding terrestrial environment through mechanisms such as well water consumption, farming, gardening, and/or living on dirt floors.

Streptomyces are a ubiquitous soil bacterial genus that have large genomes and produce a variety of secondary metabolites, including compounds that cause mitochondrial defects.⁸ Evidence suggests that PD-related toxicants cause oxidative stress and mitochondrial dysfunction, which can lead to parkinsonism in animals.⁹⁻¹¹ In previous work, we reported that a bacterial metabolite produced by Streptomyces venezuelae caused age- and dose-dependent dopamine (DA) neurodegeneration in Caenorhabditis elegans and dose-dependent degeneration of human DA producing SH-SY5Y cells.¹² Thus, this metabolite might represent a

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Abbreviations: 6-OHDA, 6-hydroxydopamine; α-syn, alpha synuclein; DA, dopamine; DNIHB, D-beta hydroxybutyrate; EtAc, ethyl acetate; EV, empty vector; GFP, green fluorescent protein; PD, Parkinson’s disease; RNAi, RNA interference; ROS, reactive oxygen species; UPR⁸⁵, endoplasmic reticulum unfolded protein response; UPR⁸⁶, mitochondrial unfolded protein response; UPS, ubiquitin proteasome system

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previously uncharacterized environmental contributor to neurodegeneration.

Here, we extend the mechanistic analysis of this novel environmental effector of neurodegeneration to report that exposure to the *S. venezuelae* metabolite causes excessive production of reactive oxygen species (ROS) in *C. elegans*, as shown by a cellular reporter for superoxide dismutase and an *in vitro* biochemical assay. Likewise, the mitochondrial unfolded protein response (UPR\textsuperscript{mt}) pathway was upregulated and adenosine triphosphate (ATP) production impaired in response to metabolite exposure. In combinational studies using additional chemical and genetic modifiers associated with PD, we determined that metabolite exposure enhanced susceptibility to cell death. Moreover, we discerned that the mechanism of action involves targeting of mitochondrial complex I, and that antioxidant treatment rescues DA neurodegeneration. Taken together, these data provide a plausible underlying mechanism involved in *S. venezuelae* metabolite-induced toxicity.

**Results**

*S. venezuelae* metabolite exposure causes oxidative stress in *C. elegans*. As previously reported,\textsuperscript{11} the neurotoxic compound under investigation is a small secondary product (molecular weight <300) isolated following growth of *S. venezuelae* to the stationary phase in liquid culture where the compound is present in spent media. The *S. venezuelae* conditioned medium was extracted in dichloromethane (DCM), and ethyl acetate (EtAc) solvent was used to reconstitute the compound following partitioning, indicating that it is amphipathic. We have calculated an almost 100% recovery rate from this extraction (data not shown). Hereafter, we use the term metabolite to refer to this compound. EtAc is used as a negative (solvent) control in all experiments and does not cause a significant DA neurodegeneration.

To determine whether the metabolite increases ROS production *in vivo*, we examined *C. elegans* expressing an established oxidative stress-inducible reporter, sod-3::GFP, where green fluorescent protein (GFP) is driven under the endogenous sod-3 gene promoter.\textsuperscript{13} sod-3 encodes a mitochondrial superoxide dismutase enzyme, which is thought to protect against oxidative stress. Worms treated with the metabolite exhibited a significant upregulation of sod-3::GFP expression compared with EtAc solvent control (Figures 1a–d). RNA interference (RNAi) depletion of daf-2, the *C. elegans* insulin/IGF receptor ortholog, was used as a positive control\textsuperscript{14} (Figures 1a and d).

ROS production in response to the metabolite was also examined in *C. elegans* using an *ex vivo* 2,7-dichloro-fluorescein diacetate (DCF-DA) assay.\textsuperscript{15} Worms treated with either the *S. venezuelae* metabolite, 100\&\mu M parquat (positive control), or EtAc solvent were analyzed at day 7, day 10, and day 12 of exposure because DA neurodegeneration is observed following metabolite exposure at these time points.\textsuperscript{12} The results showed significantly increased ROS production in metabolite- and parquat-exposed worms at all days analyzed. Figure 1e displays day 7 data (days 10 and 12 are not shown).

Previously, we reported that exposure to the *S. venezuelae* metabolite caused DA neurodegeneration.\textsuperscript{12} Treatment with 1 mM probucol, an anti-oxidant, fully rescued metabolite-induced DA neurodegeneration (Figures 1f–j). The protection by probucol indicates that free radical generation contributes to *S. venezuelae* metabolite toxicity. Thus, these data suggest that the *S. venezuelae* metabolite induces oxidative stress that, in turn, contributes to neuronal cell death.

Because sod-3 is under the direct control of DAF-16, we sought to determine whether the DAF-16 transcription factor could be induced to translocate to the nucleus in response to metabolite treatment.\textsuperscript{16} When compared with solvent treatment alone, we found that DAF-16 significantly accumulates within nuclei when animals are treated with metabolite or challenged with daf-2 knockdown (Figures 2a and b). The nuclear accumulation observed could be related to increased ROS, because DAF-16 is a known stress-associated transcription factor induced by ROS, however, DAF-16 also responds to other stressors.\textsuperscript{17}

In mammals, NRF-2 is the major ROS and detoxification transcription factor.\textsuperscript{18} The *C. elegans* NRF-2 homolog, SKN-1, can be translocated to the nucleus by a variety of sources including pathogens and ROS.\textsuperscript{19,20} However, when put to direct observation, SKN-1 failed to change its intracellular localization in response to metabolite treatment (data not shown), indicating that the ROS produced by the metabolite in *C. elegans* may not be sensed by the SKN-1 machinery.

*S. venezuelae* metabolite toxicity causes mitochondrial dysfunction. Mitochondria are known to be a major source of ROS and perturbations that affect their metabolic activity can further promote the build-up of ROS and lead to complexities within the mitochondria such as an accumulation of misfolded proteins.\textsuperscript{21} This subsequently triggers a stress-response pathway referred to as the UPR\textsuperscript{mt}, which activates transcription of mitochondrial chaperone genes to promote protein homeostasis. In *C. elegans*, this can be monitored in a strain expressing hsp-6::GFP,\textsuperscript{22} where HSP-6 is a nuclear encoded mitochondrial chaperone and GFP is driven by the hsp-6 promoter. *S. venezuelae* metabolite exposure caused an upregulation of hsp-6::GFP in day 4 worms when compared with solvent control (Figures 3a–c). RNAi depletion of mev-1, a *C. elegans* ortholog of a mitochondrial electron transport chain complex II subunit, was used as a positive control for increased hsp-6 activation (Figure 3a).

To investigate whether the *S. venezuelae* metabolite impacts mitochondrial respiratory chain activity (complex I–IV), ATP levels were measured in *C. elegans* extracts treated with either EtAc solvent control or *S. venezuelae* metabolite. A metabolic product of the neurotoxin MPTP [1-methyl-4-phenylpyridinium (MPP \textdagger)] which was shown to significantly decrease ATP production in a previous *C. elegans* study, was used as a positive control.\textsuperscript{24} Our results showed that worms exposed to the *S. venezuelae* metabolite or MPP \textdagger displayed significantly lower overall levels of ATP as compared with the solvent control (Figure 3d). Taken together, exposure to the *S. venezuelae* metabolite caused reduction in ATP levels, upregulation of
Bacterial metabolite causes mitochondrial dysfunction

A Ray et al

3

UPR<sup>mt</sup>, and production of ROS, revealing a role in mitochondrial dysfunction. These properties of the <i>S. venezuelae</i> metabolite are mechanistically similar to other environmental neurotoxins such as rotenone and paraquat.9,11

**Figure 1** <i>S. venezuelae</i> metabolite causes oxidative stress in <i>C. elegans</i>. (a) <i>S. venezuelae</i> (<i>S. ven</i>) metabolite caused an upregulation of sod-3::GFP expression, an indicator of oxidative stress, in empty vector (EV) RNAi-treated worms when compared with worms exposed to solvent only, as quantitated using pixel intensities as described in (b–d). When compared with metabolite exposure, RNAi knockdown of daf-2, used as a positive control, expressed similar levels of sod-3::GFP. Values are the mean ± S.D. of 3 experiments where 30 animals were analyzed per replicate (*<i>P</i> < 0.05; **<i>P</i> < 0.01; one-way ANOVA). The values were normalized to the untreated solvent control. (b–d) Representative worm images for each of the treatments described in (a) where pixel intensities were measured in a 100 × 100 µm region at the anterior bulb of the pharynx. The white box shows the region of GFP measured in all animals. (e) <i>S. venezuelae</i> metabolite and 100 µM paraquat (positive control) significantly increased the amount of intracellular ROS compared with solvent control. Worms were evaluated using a DCF-DA assay by examining extracts (*<i>P</i> < 0.01; one-way ANOVA; n = 3 independent experiments). The values were normalized to the untreated solvent control. (f) Treatment with 1 mM probucol (dissolved in ethanol), an anti-oxidant, significantly rescued the <i>S. venezuelae</i>-induced neurotoxicity in DA neurons compared with metabolite treatment alone (*<i>P</i> < 0.01; Student’s t-test; n = 3 independent experiments). (g–j) Representative images of the probucol experiment described in (g). All <i>C. elegans</i> (strain BY200) express GFP specifically in the six anterior DA neurons. In all images, large arrowheads show intact dopaminergic neuron cell bodies. Arrows indicate areas where dopaminergic neurons have degenerated. Small arrowheads indicate cell body degeneration. (g) Exposure to EtAc and ethanol (solvent for probucol) did not result in DA neuron loss. (h) The addition of probucol did not cause neurotoxicity, as evidenced by intact DA neurons. (i) <i>S. venezuelae</i> metabolite exposure caused substantial degeneration of cell processes, as displayed throughout the processes. Further, two of the cell bodies are degenerating and one is missing in this representative worm. (j) Probucol rescues <i>S. venezuelae</i>-induced DA neuronal toxicity, as shown in this <i>C. elegans</i> example. Magnification bars = 50 µm

<i>S. venezuelae</i> metabolite toxicity involves mitochondria complex I. Previously, it was demonstrated that the environmental toxins, rotenone and paraquat, as well as the experimental toxin model, 6-hydroxydopamine (6-OHDA), inhibit complex I of the mitochondria.11,25,26 Our results indicating that exposure to the <i>S. venezuelae</i> metabolite caused impairment of mitochondrial function led us to further investigate whether this metabolite targets complex I. Here, we explored whether two chemicals that rescue complex I deficiency would provide protection against <i>S. venezuelae</i>-induced DA neurodegeneration. Riboflavin is an activator of mitochondrial complex I (NADH dehydrogenase) and D-beta-hydroxybutyrate (DHB) is a complex II activator.
Bacterial metabolite causes mitochondrial dysfunction

A Ray et al

To further investigate the involvement of mitochondrial electron transport chain, we performed RNAi knockdown of mitochondrial genes, gas-1 and nuo-1 (subunits of complex I) and mev-1 (subunit of complex II) specifically in DA neurons, and assayed for neurodegeneration using DA neuron-specific RNAi.30 Depletion of these gene products without metabolite did not result in DA neurodegeneration (Figure 4f). Notably, addition of metabolite also did not cause DA neurodegeneration in either of the complex I gene knockdown conditions while the metabolite did cause degeneration in mock, empty vector (EV) RNAi conditions. Therefore, this experiment provides evidence that complex I is impaired by the metabolite. Conversely, knockdown of the complex II subunit, mev-1, resulted in a significant neurodegeneration compared with control (Figure 4f). Moreover, RNAi knockdown of complex I genes resulted in rescue of metabolite-induced DA neurodegeneration compared with mock RNAi treated with metabolite (Figure 4f). These results, together with the neuroprotection afforded by riboflavin and D/HB, suggest that complex I is involved in S. venezuelae metabolite-induced neurotoxicity. This complex I-specific effect could be due to the excessive production of ROS following metabolite exposure.

Mitochondrial complex I function may be decreased by \(\alpha\)-syn,31 a protein that when overexpressed or mutated, can lead to familial PD. Overexpression of \(\alpha\)-syn is often used to induce neurodegeneration in animal models. In C. elegans, dopaminergic neurons undergo age-dependent neurodegeneration following human \(\alpha\)-syn overexpression.32 We exposed transgenic \(\alpha\)-syn worms to the metabolite and discovered that the DA neurons showed enhanced degeneration to metabolite treatment (Figures 5a–c). Furthermore, \(\alpha\)-syn-expressing worms exposed to metabolite displayed significantly more DA neurodegeneration than metabolite-treated animals expressing GFP only in DA neurons (Figure 5a). Thus, the compromised genetic background in the DA neurons of these animals rendered them more susceptible to secondary neurotoxicity via metabolite exposure.

In post-mortem human brains, mitochondrial accumulated \(\alpha\)-syn was shown to interfere with complex I.31 Furthermore, in vitro studies demonstrated that \(\alpha\)-syn directly associates with mitochondrial membranes and causes mitochondrial fragmentation.33 In this regard, we sought to evaluate whether there was an additive effect from the metabolite in neurons compromised from both \(\alpha\)-syn overexpression and reduced complex I or II function through RNAi knockdown. Without metabolite susceptibility to DA neurodegeneration was significantly enhanced following RNAi knockdown of any one of these three genes beginning at day 6 compared with EV control in \(\alpha\)-syn-expressing worms (Figure 5d). Metabolite exposure of \(\alpha\)-syn-expressing C. elegans that were also treated with gas-1 or mev-1 (RNAi) did not result in enhanced DA neurodegeneration (Figure 5d). However, metabolite exposure of these worms treated with nuo-1 (RNAi) resulted in enhanced neurodegeneration compared with nuo-1 (RNAi) solvent control worms. Also, DA neurodegeneration was exacerbated by nuo-1 (RNAi) versus EV control when treated with metabolite (Figure 5d). These data suggest that \(\alpha\)-syn-expressing DA neurons treated with metabolite treated are more vulnerable to nuo-1 knockdown. In general, mechanisms associated with \(\alpha\)-syn and complex I gene interactions are not fully understood. Thus, the effect observed with our results in Figure 5d (versus Figure 4f) could be a result of direct or indirect interactions between metabolite, \(\alpha\)-syn and mitochondrial components.

Another familial form of PD, mutation in LRRK2 (G2019S), has been shown to impact mitochondrial function whereby mitochondrial membrane potential and intracellular ATP production were impaired.34 Studies in nematodes and mice have shown that this LRRK2 mutation can increase kinase activity and cause neuronal toxicity.35 Thus, we examined the effect of metabolite using the LRRK2 G2019S mutation in C. elegans. The metabolite significantly enhanced DA neurotoxicity compared with solvent control (Figure 5e).
When exposed to metabolite, LRRK-2 G2019S expressing worms displayed significantly more DA neurodegeneration than animals expressing GFP only in DA neurons (Figure 5e).

**S. venezuelae** metabolite potentiates 6-OHDA or rotenone-induced DA neurodegeneration. Epidemiological studies indicate that idiopathic parkinsonism could result from a combination of various risk factors. Therefore, we asked whether exposure to toxicants used in PD models that induce mitochondrial dysfunction and oxidative stress, such as 6-OHDA and rotenone, along with the **S. venezuelae** metabolite, would result in enhanced DA neurodegeneration. 6-OHDA inhibits mitochondrial complexes I and IV, whereas rotenone displays specificity for complex I.⁵,²⁵

We first examined a combination of 6-OHDA and metabolite. *C. elegans* were incubated with the **S. venezuelae** metabolite for half the standard exposure time to enhance visualizing neuronal vulnerability to a secondary stressor, such as 6-OHDA, while not appreciably causing neurodegeneration with metabolite alone. In this metabolite-only exposure paradigm, ~ 80% of the population still displayed normal DA neurons (Figures 6a and b). Nematodes were exposed to the metabolite (or EtAc solvent control) and exposed to 6-OHDA for 1 h at late larval stage 4. Here, we observed that 48 h post 6-OHDA treatment, worms exposed to **S. venezuelae** metabolite and 6-OHDA did not show a significant degeneration compared with worms treated with 6-OHDA alone. However, after 72 h, this same treatment provided a significant degeneration with respect to the controls (Figures 6b–e).

We further investigated the combined effect of rotenone, a known complex I inhibitor, and **S. venezuelae** metabolite. Unlike the 6-OHDA regimen, worms were continuously exposed to rotenone or DMSO solvent from the L4 stage and analyzed at day 10 (Figure 6f). Co-exposure of the metabolite with rotenone enhanced DA neurodegeneration compared with metabolite or rotenone alone (Figures 6g–i).

Together, these results suggest that mitochondrial respiratory chain activity is sensitive to the metabolite-induced toxicity. These data also suggest that exposure to more than one environmental/chemical risk factor has an additive effect on DA neurodegeneration.
Discussion

We previously reported an initial description of a highly stable and small molecular metabolite from *S. venezuelae* that caused *C. elegans* dopaminergic neurodegeneration and death of human SH-SY5Y neurons. The impetus for that nascent work stemmed from our interest in what type of hypothetical and more common exposure could, in combination with misfortune in the genetic lottery, potentially account for the prevalence of PD – the second most common neurodegenerative disorder. However, while multi-hit hypotheses are attractive in clinically defining disease etiology, there remains little functional evidence for such scenarios. In this more mechanistic investigation, we exploit the advantages afforded through *C. elegans* research to combine genetic and toxicological analysis in a well-defined system that facilitates rapid and reproducible evaluation of neurodegeneration. We determined that the neurotoxic *S. venezuelae* metabolite caused excessive production of free oxygen radicals, upregulated the UPRmt, and impaired mitochondrial complex I activity (Figure 7).

Figure 4 The *S. venezuelae* metabolite impacts mitochondrial complex I. (a–e) Metabolite-induced DA neurotoxicity was rescued by riboflavin and D/hHB, drugs that rescue mitochondria complex I deficiency. (a) *S. venezuelae* and 1 µM riboflavin significantly rescued DA neurons compared with the metabolite alone. (b) When 50 mM D/hHB is co-administered with the metabolite, *C. elegans* DA neurons are rescued from neurotoxicity. (c–e) Representative images of riboflavin and D/hHB rescuing DA neurotoxicity induced by the metabolite. All *C. elegans* (strain BY200) express GFP specifically in the six anterior DA neurons. In the images, large arrowheads show intact dopaminergic neuron cell bodies. Arrows indicate areas where dopaminergic neurons have degenerated. Small arrowheads indicate cell body degeneration. (c) Exposure to the *S. venezuelae* metabolite caused neuronal loss in this worm where five of the six DA neurons are degenerating. (d) The addition of riboflavin completely rescued DA neurons in an animal exposed to the metabolite. (e) D/hHB also rescued *S. venezuelae*-induced DA neuronal toxicity, as shown in this *C. elegans* example. Magnification bar = 50 µm. (f) RNAi knockdown of complex I component gas-1 and nuo-1 resulted in rescue of DA neurodegeneration when treated with metabolite while complex II component mev-1 and EV RNAi showed enhanced DA neurodegeneration with metabolite exposure (P-values < 0.05; one-way ANOVA; n = 90 worms). *C. elegans* (strain UA202) were analyzed at day 12 where data were analyzed as the mean ± S.D.
Defects in mitochondria have long been known to contribute to neurodegeneration. Mitochondrial damage is more deleterious in neurons than other cell types as they are non-mitotic, have high metabolic activity, and low antioxidant capacity. Using an oxidative stress inducible reporter, sod-3::GFP, we determined that the S. venezuelae metabolite increased ROS in vivo by measuring GFP levels within intestinal cells. These data are correlated with ROS production within whole animal extracts, as assayed biochemically using DCF-DA, where ROS was also upregulated in response to metabolite exposure. Finally, since we were interested in knowing whether the neurotoxicity associated with the metabolite was related to ROS, we treated C. elegans with an antioxidant, probucol, and assayed for DA neurodegeneration following metabolite exposure. Probucol protected these animals from neurodegeneration, thus providing further evidence that the metabolite elicits its toxicity at least partly through increased ROS production.

Figure 5  Gene and environment interaction enhances DA neurodegeneration. A combination of exposure to S. venezuelae and overexpression of known Parkinson’s gene products, α-syn (a–d) or LRRK2 G2019S (e) enhances DA neurodegeneration. (a) C. elegans expressing GFP alone exhibit metabolite-induced age-dependent DA neurodegeneration that is evident when examining day 6 versus day 8 animals. Worms overexpressing α-syn display age-dependent DA neurodegeneration and are also more susceptible to metabolite-induced DA neurotoxicity when compared with populations of α-syn-expressing worms treated with solvent only. These data are represented as the mean ± S.D. n = 90 per data point (*P < 0.05 by one-way ANOVA). (b and c) Representative images of C. elegans (strain UA44) expressing α-syn and GFP specifically in the six anterior DA neurons in solvent (b) or in combination with metabolite exposure (c). In the images, large arrowheads show intact dopaminergic neuron cell bodies. Arrows indicate areas where dopaminergic neurons have degenerated. (d) RNAi knockdown of gas-1, nuo-1, and mev-1 showed enhanced α-syn-induced DA neurodegeneration compared with α-syn alone without metabolite exposure. C. elegans (strain UA196) were analyzed at day 6 where data were analyzed as the mean ± S.D. After metabolite exposure, nuo-1 RNAi-treated worms showed a significant sensitivity in worms expressing α-syn compared with EV (RNAi) metabolite-treated worms and nuo-1 (RNAi) solvent control worms, whereas RNAi of gas-1 and mev-1 failed to cause a significant degeneration with metabolite treatment when compared with untreated controls (*P < 0.05; one-way ANOVA). (e) Metabolite-treated worms expressing α-syn alone do not display a significant DA neurodegeneration when compared with solvent control at 7 days of exposure. Susceptibility to metabolite-induced DA neurotoxicity is enhanced when C. elegans overexpress human LRRK2 G2019S when compared with populations of worms treated with solvent only. Data are represented as the mean ± S.D., n = 90 per independent transgenic line; where three separate transgenic lines were analyzed (*P < 0.05 by one-way ANOVA). Magnification bar = 50 μm.
An accumulation of ROS can trigger the UPR^mt, a stress-response pathway that activates transcription of mitochondrial chaperone genes to promote protein homeostasis. The metabolite-induced activation of the UPR^mt that we observed using a worm strain expressing hsp-6::GFP was suggestive of a disturbance of mitochondrial homeostasis. Importantly, we previously demonstrated that the metabolite failed to activate hsp-4::GFP, an indicator of endoplasmic reticulum-induced UPR (UPR^ER). Thus, our findings suggest that the UPR response triggered by S. venezuelae metabolite is mitochondrial specific. The mitochondrial protein-folding environment can be perturbed by any changes in the organelle structure, excess production of free radicals, and/or improper function of the electron transport chain. The neurotoxicity we document in C. elegans is coincident with increased ROS generation and depletion of ATP levels, both of which imply mitochondrial dysfunction (Figure 7).

More detailed investigation of the mitochondrial impairment caused by the S. venezuelae metabolite highlighted impairment of complex I. Using activators of mitochondrial complex I, riboflavin and D/HB, DA neurodegeneration resulting from the metabolite was rescued. Riboflavin has been shown to improve both complex I and complex IV (cytochrome c oxidase) activity whereas D/HB rescues complex I through complex II. D/HB has been also shown to protect mouse DA neurons by mitigating the detrimental effects of complex I inhibition. Worms treated with RNAi to knockdown complex I genes and the metabolite did not display neurodegeneration, further suggesting that the metabolite might target mitochondrial complex I. However, further investigation will include the direct measurement of complex I–IV enzymatic activities to confirm the direct target, or targets, of the metabolite. Currently, the compound we are utilizing in our studies could be a mixture of more than one metabolite. Efforts are underway to purify the neurotoxic molecule for future investigations.

Current literature suggests a reciprocal relationship between mitochondria and the ubiquitin proteasome system (UPS). Metabolic function of the UPS impacts the regulation of mitochondrial dynamics, wherein functional perturbations in one of these systems affect the other. More specifically, current paradigms describe in (a). (g) C. elegans co-exposed with the metabolite and 5 μM rotenone (in 0.05% DMSO) display significantly more susceptibility to DA neurodegeneration than either treatment alone (P < 0.05; one-way ANOVA; n = 90 per treatment). (c–e) Representative images of C. elegans (strain BY200) expressing GFP specifically in the six anterior DA neurons. In the images, large arrowheads show intact dopaminergic neuron cell bodies. Arrows indicate areas where dopaminergic neurons have degenerated. Small arrowheads indicate cell body degeneration. (q) A control worm exposed to 6-OHDA is missing one neuron. (b) This representative worm exposed to both rotenone and 6-OHDA was missing two neurons while another three neurons display cell body rounding.

![Figure 6](image-url) Hypersensitivity to S. venezuelae DA neurotoxicity when worms are treated with rotenone or 6-OHDA. (a) A timeline representing an experimental paradigm depicting the length of S. venezuelae metabolite exposure and 6-OHDA treatment. The abbreviations L1–L4 are the larval stages of C. elegans, while the ‘adult’ designations represent days post hatching. The 48 and 72 hr represents times, post 1 hr 6-OHDA (30 mM) treatment when DA neurons were analyzed. (b) At 48 hr after 6-OHDA treatment, co-treatment with metabolite was not significantly different from individual treatments alone. Whereas, after 72 hr, C. elegans co-exposed with the metabolite and 6-OHDA displayed significantly more susceptibility to DA neurodegeneration than either treatment alone (P < 0.05; one-way ANOVA; n = 90 per treatment). (c–e) Representative images of C. elegans (strain BY200) expressing GFP specifically in the six anterior DA neurons. In the images, large arrowheads show intact dopaminergic neuron cell bodies. Arrows indicate areas where dopaminergic neurons have degenerated. Small arrowheads indicate cell body degeneration. (c) A control worm exposed to 6-OHDA (30 mM) treatment when DA neurons were analyzed. (d) This representative worm exposed to both 6-OHDA and the metabolite is missing one neuron. (e) In this example, a worm exposed to both 6-OHDA and the metabolite is missing two neurons while another three neurons display cell body rounding, indicative of degeneration. Magnification bar = 50 μm. (f) A timeline representing the experimental paradigm for a combination of S. venezuelae metabolite and rotenone exposure scored for DA neurodegeneration. The abbreviations L1–L4 are the larval stages of C. elegans, while the ‘adult’ designations represent days post hatching. The 48 and 72 hr represents times, post 1 hr 6-OHDA (30 mM) treatment when DA neurons were analyzed. Magnification bar = 50 μm. (g) C. elegans co-exposed with the metabolite and 5 μM rotenone (in 0.05% DMSO) display significantly more susceptibility to DA neurodegeneration than either treatment alone (P < 0.05; one-way ANOVA; n = 90 per treatment). (h and i) Representative images of C. elegans (strain BY200) expressing GFP specifically in the six anterior DA neurons. In the images, large arrowheads show intact dopaminergic neuron cell bodies. Arrows indicate areas where dopaminergic neurons have degenerated. Small arrowheads indicate cell body degeneration. (h) This representative worm exposed to rotenone was missing one neuron. (i) In this representative worm exposed to both rotenone and the metabolite, all neurons display cell body rounding, indicative of degeneration. Magnification bar = 50 μm.
LRRK2 in targeting mitochondria. We have previously reported that the metabolite factors. In this regard, our results on the combined neurotoxic

Several familial forms of PD have identified gene mutations that cause mitochondrial dysfunction, including parkin, PINK1, and DJ-1; studies designed to evaluate combined impact of the metabolite with these mutations are in progress.

In considering potential mechanisms that could trigger an oxidative stress response, we discerned that the S. venezuelae metabolite caused the FOXO transcription factor protein, DAF-16, to translocate to the nucleus in a manner similar to what has been reported in response to paraquat. We conclude that this nuclear accumulation caused by the metabolite relates to increased intracellular ROS and may be under the control of a genetic program to combat pathogens, sense mitochondrial dysfunction or promote cell death.13,45–47 DAF-16, along with SKN-1, are transcription factors that work in parallel and are directly inhibited by the insulin-signaling pathway. Each transcription factor contributes to stress resistance and has a set of target genes, some of which are overlapping. It is interesting to note that following exposure to the S. venezuelae metabolite, C. elegans expressing a reporter for SKN-1 did not exhibit activation, in contrast to results reported for exposure to paraquat.48 While beyond the scope of this study, future research will explore DAF-16 targets of the metabolite.

A recent study described a genome-wide RNAi screen for gene products that were upregulated in a UPRmt readout in C. elegans following paraquat exposure.49 Considering that most, but not all cellular readouts are identical following metabolite and paraquat treatments in C. elegans, it would be interesting to examine newly identified regulators of ROS-induced UPRmt.

In conclusion, we report that the S. venezuelae metabolite shares common molecular mechanisms with other PD toxicants that cause DA neurodegeneration, such as impairment of mitochondrial complex 1 and upregulation of the UPRmt. These studies advance our understanding of DA neurodegeneration and as well provide an additional model for analysis of PD-associated pathogenesis.
Isolation and extraction of *S. venezuelae* metabolite. Spores from *S. venezuelae* strain (ARS NRRL ISP-5230) were inoculated in 5 liters of SYZ media at a density of 103 and were grown under constant exposure to partially purified *S. venezuelae* metabolite or EtAc (solvent control) until analysis. For rotenone assay, BY200 worms were washed with ddH2O three times, and treated with 30 mM 6-OHDA (Toxic Bioscience, Bristol, UK) containing 1 mM ascorbic acid, followed by gentle agitation for 1 h. Subsequently, the worms were again washed and put onto freshly made metabolite or EtAc plates until analysis. For rotenone assay, BY200 worms were exposed to the metabolite or EtAc until L4 and then transferred to plates containing metabolite, along with 5 μM of rotenone or DMSO (as a solvent control for rotenone).

**ATP measurements.** The ATP assay was performed as described before with minor modifications. Worms were grown on metabolite or EtAc and collected for assay. As a positive control, EtAc-treated worms were soaked in 1 mM MPP + (Sigma, Santa Ana, CA, USA) for 1 h, left on seeded plates for 4–5 h to recover and then collected. In all, 100 age-synchronized young adult wild-type worms were washed with M9 buffer, treated with three free-thaw cycles and boiled for 15 min to release ATP and destroy ATPase activity. Samples were then spun at 4°C, 11 000 rpm for 10 min. A Life Technologies ATP determination kit (MolecularProbes, Eugene, OR, USA, A20288), which utilizes luciferase to catalyze the formation of Glomax 20/20; Promega, Madison, WI, USA) was used to measure levels of bioluminescence. For normalization, protein levels were determined by a BCA protein assay kit (Pierce, Thermo Scientific, Rockford, IL, USA).

**Drug treatment.** A final concentration of 100 μM Paraguard (Sigma), 1 μg/ml Riboflavin (Calbiochem, EMD Bioscience, San Diego, CA, USA), 1 mM Probucol (MP Biomedicals, St. Louis, MO, USA), and 50 mM DHB (Sigma-Aldrich, St. Louis, MO, USA) were supplemented in the worm media for their respective experiments. The *S. venezuelae* metabolite or EtAc was added to these plates before use.

**Quantitative fluorescence measurements and statistics.** Worms were immobilized with 3 mM levamisole and mounted on 2% agarose pads on a microscope slide. Fluorescent microscopy was performed using a Nikon Eclipse E800 epifluorescence microscope equipped with an Endow GFP HQ filter cube (Chroma Technology, Bellows Falls, VT, USA). A Cool Snap CCD camera (Photometrics, Tucson, AZ, USA) driven by the Metamorph software (Molecular Devices) was used to acquire images. Each animal was imaged in the same region at the same magnification and exposure intensity as previously described.56 When analyzing each animal, a 100 × 100 μm box was used in the same region of the animal for the soy-3 transcriptional fusion reporter, this area was the region at the anterior bulb of the pharynx. For the hsp-6 transcriptional fusion reporter, this area was the intestinal lumen immediately posterior to the gringer of the pharynx. Pixel intensity was quantified in this manner and compiled across.
three-four separate replicates. For DAF-16 experiment, the entire animal was observed. If no nuclear accumulation could be seen, then it was scored as cytoplasmic, if some, accumulation was observed then it was scored as both, and if robust, nuclear accumulation could be observed throughout the animal then it was scored as nuclear. For the SKN-1 experiment, a dual red-green filter was used to observe emerging green fluorescence from the intestine over the background fluorescence of gut granules (which appear yellow). A similar scoring system was used to that of DAF-16. For statistical analysis, a one-way ANOVA followed by post hoc Tukey’s or Dunnnett’s test was employed for comparison. More than two data sets (Prism 6.0 software; GraphPad, La Jolla, CA, USA). For comparisons between two data sets, Student’s t-test was performed. All the experiments were performed with three independent replicates and are presented as means ± S.D. A value of P ≤ 0.05 or P ≤ 0.01 is considered as statistically significant.

Conflict of Interest

The authors declare no conflict of interest.

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Bacterial metabolite causes mitochondrial dysfunction A Ray et al

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