The Anthelmintic Drug Niclosamide and its Analogues Activate the Parkinson’s Disease Associated Protein Kinase PINK1


Abstract: Mutations in PINK1, which impair its catalytic kinase activity, are causal for autosomal recessive early onset Parkinson’s disease (PD). Various studies have indicated that the activation of PINK1 could be a useful strategy in treating neurodegenerative diseases such as PD. Herein, we show that the anthelmintic drug niclosamide and its analogues are capable of activating PINK1 in cells via reversible impairment of the mitochondrial membrane potential. Using these compounds, we demonstrate for the first time that the PINK1 serine/threonine kinase is active and detectable in primary neurons. Our findings suggest that niclosamide and its analogues are robust compounds to study the PINK1 pathway and may hold promise as a therapeutic strategy in Parkinson’s and related disorders.

Loss-of-function mutations in the genes encoding the PTEN (phosphatase and tensin homologue deleted on chromosome 10)-induced kinase 1 (PINK1) and the E3 ubiquitin ligase Parkin lead to autosomal recessive early-onset PD.[1] PINK1 is a serine/threonine protein kinase that possesses an N-terminal mitochondrial targeting sequence, a transmembrane domain, and three insertional loops within its catalytic kinase domain.[2] A large body of cell biological and biochemical analyses has linked PINK1 to the regulation of mitochondrial homeostasis.[3] Indeed, it is now understood that upon mitochondrial membrane depolarization, PINK1 becomes activated and consequently phosphorylates Parkin and ubiquitin at a conserved serine 65 (Ser65) residue. This stimulates Parkin recruitment to the mitochondria whereupon it becomes maximally active and ubiquitylates multiple substrates on the outer mitochondrial membrane to trigger degradation of damaged mitochondria via autophagy (mitophagy).[4]

The majority of PD-related PINK1 mutations abrogate its kinase activity[5] and prevent the initiation of mitophagy in cells upon mitochondrial damage leading to the accumulation of reactive oxygen species and premature neuronal loss.[6] This underlines the kinase activity of PINK1 as being critical to the prevention of neurodegeneration. Such hypothesis has been verified in Drosophila models of PINK1 in which kinase-inactive versions of PINK1 failed to rescue neurodegeneration compared to the wild-type gene.[7] This important finding highlighted the activation of PINK1 as a promising strategy for inducing and maintaining neuroprotective effects.

To date, a series of agents have been reported to efficiently activate PINK1 in various immortalized human cell lines. These could be divided into two groups; compounds that act directly as PINK1 ATP neosubstrates[8] and indirect PINK1 activators that cause the loss of the mitochondrial membrane potential (Δψm).[9]

Undoubtedly, the latter class of compounds, which include the proton ionophore, carbonyl cyanide m-chlorophenyl hydrazone (CCCP), the potassium uniporter valinomycin, or a combination of Antimycin A and Oligomycin A (O/A) have attracted more interest in the study of PINK1-signalling. Despite the promise of these agents in activating PINK1, their cellular toxicity has limited their translation to activating PINK1 in vivo. Hence, the elaboration of novel and safe (direct or indirect) activators of PINK1 is of great biological and therapeutic interest.

As indirect PINK1 activation can be triggered by the uncoupling of the mitochondria,[9] we focused our search of small molecule PINK1 activators on niclosamide (Figure 1A), an anthelmintic drug that was previously reported for its potential in treating myeloma through the uncoupling of oxidative phosphorylation in the mitochondria.[10] Given that niclosamide has been used for a long time as a safe anthelmintic drug[11] and studied in vivo with no apparent severe side effects,[12] we were encouraged to explore the activation of PINK1 by this clinical agent. For this, untagged Parkin was expressed in both wild-type and PINK1 knockout HeLa cells generated by CRISPR/Cas9 technology.[13] The cells were treated with different concentrations of niclosamide (0.2-20 µM) for 40 min, DMSO or 10 µM Antimycin A /1 µM Oligomycin A (O/A) for 3 h. The cell lysates were immunoblotted with an anti-phospho-Parkin Ser65 antibody to monitor PINK1 activity.[14] Niclosamide has been shown to uncouple the mitochondria preventing the creation of adenosine triphosphate (ATP).[12] To monitor for the ability of niclosamide...
and A/O to induce mitochondrial uncoupling, we probed for the cleavage of the mitochondrial protein, optic atrophy protein 1 (OPA1), that is catalyzed by the zinc metalloprotease, OMA 1, upon mitochondrial membrane depolarization in cells.\cite{14} We observed mild activation of PINK1 as judged by Parkin Ser65 phosphorylation at 0.2 μM and more striking activation at 2 μM or higher concentrations of niclosamide comparable to that induced by A/O treatment at 3 h (Figures 1B). This was associated with ubiquitylation of the mitochondrial Fe/S domain containing protein, CISD1, that is a readout of Parkin ubiquitin E3 ligase activity (Figure 1B).\cite{15}

![Figure 1. Niclosamide activates PINK1 in HeLa cells. A. Chemical structure of niclosamide. B. Niclosamide dose-response analysis. Wildtype (WT) and PINK1 knockout (PINK1 KO) HeLa cells transfected with Parkin were stimulated with either a combination of A/O for 3 h or with different concentrations (0.2, 0.8, 2, 8, 20 μM) of niclosamide (Niclo) for 40 min. Parkin Ser65 phosphorylation (pS65Parkin), Parkin, Full length OPA1 (F/L), Cleaved OPA1, ubiquitylated CISD1 (CISD1-Ub) and CISD1 were detected by immunoblotting. GAPDH was used as a loading control.](image1)

Importantly, the ability of niclosamide as well as A/O to induce Parkin Ser65 phosphorylation and CISD1 ubiquitylation was abolished in PINK1 knockout cells (Figure 1B). However, their ability to induce uncoupling was not affected as judged by cleavage of OPA1 (Figure 1B). Under similar transfection and cell conditions we next undertook a time-course analysis of Parkin Ser65 phosphorylation and CISD1 ubiquitylation in the presence of 20 μM niclosamide. We observed robust niclosamide induced Parkin Ser65 phosphorylation after 20 min of treatment (Supplementary Figures 1) associated with ubiquitylation of CISD1 (Supplementary Figure 1A). In vitro kinase assays of PINK1 in the presence or absence of niclosamide showed no evidence for direct activation of PINK1 by the compound (data not shown).

Facile chemical modification of the salicylaldehyde scaffold of niclosamide enabled synthesis of three brominated analogues known to exert pharmacological efficacy, AM85 (Dibromsalan), AM86 (Tribromsalan) and AM87 (Metabromsalan) (Figure 2A) (see Supporting Information).\cite{16} To compare the effects of these niclosamide analogues on PINK1 activation, HeLa cells were treated with 20 μM of niclosamide or AM85-AM87 for 40 mins. Interestingly, less potent PINK1 activators AM86 and AM87 induced similar cleavage of OPA1 to A/O and niclosamide indicating broadly similar effects on the ∆ψm (Figure 2B). We next undertook a dose-response analysis of AM85 on PINK1 activation and observed robust Parkin Ser65 phosphorylation and CISD1 ubiquitylation at 2, 8 and 20 μM but not at lower concentrations (Figures 2C). CISD1 ubiquitylation, was also confirmed by pull down of ubiquitylated substrates with HALO-UBAUBQLN1 resin as previously described (Supplementary Figure 2A).\cite{14}

Furthermore, time-course analysis of AM85 demonstrated Parkin Ser65 phosphorylation and CISD1 ubiquitylation at 40 min but not earlier time points that were previously observed for niclosamide (Supplementary Figures 2B-C).

To quantify the degree of mitochondrial uncoupling between AM85 and niclosamide, we performed FACS analysis on HeLa cells treated with 20 μM of niclosamide and AM85 for 40 min. Cells were incubated with CMX ROS, which is a cell permeable probe that accumulates in the active mitochondria and emits at 599 nm.\cite{17} Interestingly, both niclosamide and AM85 promoted significant mitochondrial membrane depolarization comparable with 3 h A/O-treated cells (Figure 2D and Supplementary Figure 2d) in agreement with OPA1 cleavage shown above (Figures 1B and 2B-C). Critically, the mitochondrial depolarization effect induced by both niclosamide and AM85 was reversible since no reduction in ROS was detected after compound wash-out (Figure 2E). FACS analysis also demonstrated no significant toxicity under the compound conditions used for niclosamide and AM85 compared to DMSO (Supplementary Figure 3).
or with 1h of niclosamide (Niclo, red) and AM85 (blue). Values are normalized to the vehicle, DMSO (black). E. Quantification of CMXRos Fluorescence Intensity (arbitrary units, a.u.) for HeLa cells subjected to drug wash out, after treatment with A/O (green), niclosamide (Niclo, red), AM85 (blue), normalized to the vehicle, DMSO (black). Bars represent the average ratio ± SEM of three independent experiments. **p < 0.01, one-way ANOVA followed by Bonferroni post-test correction.

We next determined the ability of niclosamide and AM85 to activate PINK1 in cells of pathophysiological relevance to PD. To date no studies have assessed PINK1 activity in primary neurons under conditions where the PINK1 and Parkin are expressed at endogenous levels. Marked neuronal loss and Lewy body accumulation occurs in the frontal cortex particularly the anterior cingulate gyrus in advancing Parkinson’s disease.[18] Therefore, we studied primary cortical neurons derived from E16.5 embryos.

We initially evaluated Parkin expression in cortical neurons at various time points from 3-21 days in vitro (DIV) (Figure 3A). We strikingly observed an increase in Parkin expression during cortical neuronal growth in vitro that paralleled the expression of the pre-synaptic and post-synaptic proteins, synaptophysin and PSD95 respectively (Figure 3A).

We next undertook a time-course analysis of 12 DIV neurons treated with A/O and observed Parkin Ser65 phosphorylation occurring at 10 min of stimulation and becoming maximal by 1 h of stimulation and sustained for 9 h (Figure 3B). Furthermore, we observed significant CISD1 ubiquitylation in 12 DIV and 21 DIV neurons stimulated with A/O for 3 h but not in 5 DIV or 7 DIV neurons (Figure 3C). We next tested the ability of niclosamide and AM85 to activate PINK1 in 21 DIV neurons. Neurons were treated with 30 µM of niclosamide or AM85 for 1 h and this led to increased Parkin Ser65 phosphorylation (Figure 3D). Interestingly, AM85 exerted a stronger effect on Parkin Ser65 phosphorylation than niclosamide and this was also confirmed by CISD1 ubiquitylation in neurons under similar conditions (Figure 3E). This difference was not explained by differences in uncoupling since both drugs had a similar effect on mitochondrial depolarization as assessed directly by FACS or indirectly by cleavage of OPA1 (Figures 3F-G).

In summary, we report the discovery that the anthelminthic drug, niclosamide, and its analogue, AM85, can activate PINK1 in cells. Notably, we detected for the first time PINK1-Parkin pathway activation in neurons and demonstrated that it can be triggered by small molecules. Additionally, we showed that the induction of mitochondrial depolarization is capable of activating endogenous PINK1 protein in neurons leading to Parkin Ser65 phosphorylation and ubiquitylation of its mitochondrial substrate CISD1. The mechanism of action of niclosamide and AM85 appears to be indirect and mediated by their mitochondrial uncoupling properties although this is not sufficient to explain the greater potency of AM85 in neurons. Niclosamide has been used safely in humans for over half a century to treat helminth infections and human cancers, as well as rheumatoid arthritis.[19] Our data suggests that niclosamide and/or its analogues could have therapeutic benefit in slowing down Parkinson’s disease progression through the activation of PINK1. Further in vivo studies in appropriate PD models are warranted to test this hypothesis.
Experimental Section

Immunoblotting and immunoprecipitation

Tissues, primary cortical neurons or Hela cells were sonicated in lysis buffer containing 50 mM Tris–HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 1% (w/v) Triton, 1 mM sodium orthovanadate, 10 mM sodium glycerophosphate, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 0.25 M sucrose, 1 mM benzamidine, 0.1 mM PMSF and protease inhibitor cocktail (Roche). Following the sonication, lysates were incubated for 30 min on ice. Samples were spun at 20,800 x g in an Eppendorf 5417R centrifuge for 30 min. Supernatants were collected and protein concentration was determined using the Bradford kit (Pierce). Samples were acquired using a BD FACS Canto and in drug absence. All harvested cells were incubated for 5 min on ice after centrifugation for 30 min at 4 °C. Detection was performed using HRP-conjugated secondary antibodies and enhanced chemiluminescence reagent.

Ubiquitin enrichment

For ubiquitylated protein capture, 400 μg of extract was used for pull down with HALO-UABUBQLN1 resin as described previously[5]. Halo-tagged UBDs were incubated with 200 μl of the HaloLink resin (Promega) in binding buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.05% NP-40) overnight at 4 °C. 20 μl of Halo Tube beads were added to neuronal or tissue lysates and were incubated for 4 h at 4 °C. The beads were washed three times with lysis buffer containing 0.25 M NaCl and eluted by resuspending in 20 μl of 1× LDS sample buffer with 1mM DTT.

Flow Cytometry Analysis of mitochondrial membrane potential

Hela Cells were incubated with 20 μM niclosamide (manufacturer), AM65 (manufacturer) for 40 min before trypsinization and collection. Oligomycin (manufacturer) and Antimycin (manufacturer) 20 μM were used as positive control and incubated for 3 h before samples harvest. DMSO (Sigma Aldrich) at the same concentration was used as control. After 10 min from start of drug treatment, cells were treated with 100 nM Mito Tracker CMXRos (Cell Signaling Technology) for 30 min on directly on wells. Drug Washout was performed incubating trypsinized floating cells with 100 nM Mito Tracker CMXRos (Cell Signaling Technology) for 30 min at 37 °C in drug absence. All harvested cells were incubated for 5 min on ice after CMXRos incubation and then centrifuged and washed two times with a 1% BSA/PBS solution. Finally, cells were treated with DAPI (1:200) solution (1 mg/ml DAPI, 50 μg/ml RNaseA in 1% BSA/PBS) and transferred to FACS tubes for analysis. Samples were acquired using a BD FACS Canto and the results analysed using FlowJo software.

Statistical analysis

Statistical analysis of groups with normal distributions was performed using One-way ANOVA or two-way ANOVA followed by Holm-Sidak or Bonferroni post-test. Differences among groups were considered statistically significant when p < 0.05. Data throughout the text are reported as average values ± SEM unless otherwise specified.

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Keywords: PINK1 • Parkin • Mitochondria • Niclosamide • Parkinson’s disease

References


COMMUNICATION

Catalytically impaired PINK1 causes autosomal recessive early onset Parkinson’s disease (PD). Herein, we show that niclosamide and its analogues activate PINK1 via reversible impairment of the mitochondrial membrane potential. Using these compounds, we demonstrate for the first time that the PINK1 pathway is active and detectable in primary neurons. The reported compounds are thus robust tools for studying PINK1-signalling.

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I. Antibodies and reagents

The following primary antibodies were used: mouse monoclonal antibodies against Parkin (Santa Cruz), βI-tubulin (Sigma), β-actin (Sigma), GAPDH (Santa Cruz), PSD95 (Cell Signaling), synaptophysin (cell signalling), CISD1 (Proteintech). Horseradish-peroxidase (HRP)-conjugated secondary antibodies (Sigma) were used. Anti-Parkin phospho-Ser65 rabbit monoclonal antibody was raised by Epitomics in collaboration with the Michael J Fox Foundation for Research.

Stock solutions of niclosamide (Sigma), Antimycin A (Sigma) Oligomycin A (Sigma) were used for experiments in vitro. Unless otherwise specified, general reagents and chemicals were from Sigma and cell culture reagents were from Invitrogen.
II. Cell culture
HeLa cells stably expressing untagged Parkin WT and PINK 1 KO, were cultured using DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 10% FBS, 2 mM L-glutamine, 1 × penicillin/streptomycin. To uncouple mitochondria, cells were treated with 10 µM Antimycin (Sigma) and 1 µM Oligomycin dissolved in DMSO for 3 h. To express Parkin, cell transfections were performed using polyethylenimine (Polysciences) according to the manufacturer’s instruction. Primary cortical neurons were isolated from the brain of WT embryos of either sex at E16.5. Embryonic cortices were collected in HBSS, and cells were dissociated by incubation with trypsin (GIBCO) at 37°C. Cells were then diluted in Neurobasal medium containing B27 supplement, Glutamax, penicillin/streptomycin and plated at a density of 6.0 × 106 cells/well on 6-well plates coated with 0.1 mg/ml poly-L-lysine (PLL; Sigma). Neurons were cultured at 37 °C in a humidified incubator with 5% CO2. Every 7 days, the medium was replaced with fresh medium containing B27. Neurons were treated with 30 µM niclosamide and analogues for 1h at 37 °C.

III. Synthesis of AM85-AM87 compounds
To a stirring solution of the relevant salicylic acid (1 eq.) in dry THF, PyBop (1.2 eq.) and triethylamine (3 eq.) were added. After 10 min, the relevant aniline (1.5 eq.) was added to the cloudy white suspension and stirred at room temperature for 12 h. The reaction mixture was then concentrated under reduced pressure to afford a yellow oil, which was subsequently dissolved in water and extracted in ethyl acetate. The combined organic layers were dried (MgSO4), filtered and concentrated under reduced pressure to form a viscous orange oil. Flash column chromatography employing ethyl acetate:hexane (1:1) was used to yield the desired AM compounds as off-white solids.

5-Bromo-N-(4-bromophenyl)-2-hydroxybenzamide (AM85). Yield 10%. 1H NMR (500 MHz, DMSO): δ 11.73 (s, 1H, OH), 10.49 (s, 1H, NH), 8.03 (d, J = 2.5 Hz, 1H), 7.69 (d, J = 8.9 Hz, 2H), 7.57 (m, 3H), 6.97 (d, J = 8.8 Hz, 1H). 13C NMR (126 MHz, DMSO): δ 165.36, 157.53, 137.98, 136.31, 132.39, 131.77, 123.10, 120.92, 119.97, 116.46, 110.59. HRMS m/z [M+H]+ calcd. for C13H10NO2Br2: 369.9078, found: 369.9067. Anal. Calcd for C13H9NO2Br2: C, 42.08; H, 2.44; N, 3.77. Found: C, 41.93; H, 2.61; N, 3.93.

3,5-Dibromo-N-(4-bromophenyl)-2-hydroxybenzamide (AM86). Yield 9%. 1H NMR (500 MHz, DMSO): δ 12.75 (s, 1H, OH), 10.71 (s, 1H, NH), 8.26 (s, 1H), 8.01 (s, 1H), 7.67 (d, J = 8.6 Hz, 2H), 7.59 (d, J = 8.5 Hz, 2H). 13C NMR (126 MHz, DMSO): δ 167.09, 156.71, 139.09, 137.27, 132.07, 124.06, 119.10, 117.41, 112.93, 110.39. HRMS m/z [M+H]+ calcd. for C13H9NO2Br3: 447.8183, found: 447.8184. HPLC (8 min), tR = 5.21 min, purity: 97%.

3,5-Dibromo-2-hydroxy-N-phenylbenzamide (AM87). Yield 15%. 1H NMR (500 MHz, DMSO): δ 13.01 (s, 1H, OH), 10.65 (s, 1H, NH), 8.31 (d, J = 2.2 Hz, 1H), 8.02 (d, J = 2.2 Hz, 1H), 7.68 (d, J = 7.7 Hz, 2H), 7.41 (t, J = 7.9 Hz, 2H), 7.21 (t, J = 7.4 Hz, 1H). 13C NMR (126 MHz, DMSO): δ 167.18, 156.98, 138.91, 137.87, 130.26, 129.21, 125.40, 122.23, 118.88, 113.14, 109.71. HRMS m/z [M+H]+ calcd. for C13H10NO2Br2: 369.9078, found: 369.9087. Anal. Calcd for C13H9NO2Br2: C, 42.08; H, 2.44; N, 3.77. Found: C, 41.91; H, 2.37; N, 3.71.
Supplementary 1. Niclosamide activates PINK1 after 20 minutes treatment. A) Niclosamide-induced Parkin activation is PINK1 dependent and can be detected after 20 min of *in vitro* stimulation. HeLa cells transfected with Parkin were stimulated with either a combination of antimycin A and oligomycin A (A/O) for 3 h or with 10 µM of niclosamide (Niclo) for 5, 10, 20, 40 min. Detection of Parkin S65 phosphorylation (pS65Parkin), Parkin, Full length (F/L) and cleaved OPA1, CISD1 ubiquitylation (CISD1-Ub) can be detected in niclosamide stimulated HeLa cells at different time points. GAPDH and HSP60 were used as loading controls. B) Quantitative analysis of Parkin S65 phosphorylation after niclosamide treatment at different time points (5, 10, 20, 40 min). Bars represent the average ratio ± SEM of two independent experiments and the value is a ratio between pS65 Parkin and total Parkin.
V. Supplementary figure 2

Supplementary 2. AM85 activates PINK1 and uncouples mitochondria after 40 minutes of treatment. A) CISD1 ubiquitylation after niclosamide and AM85 treatment is PINK1 dependent. Wildtype (WT) and PINK1 knockout (Pink1 KO) HeLa cells transfected with Parkin were stimulated with a combination of 10 µM antimycin A and 1 µM oligomycin A (A/O) for 3 h, 10 µM niclosamide (Niclo) and 10 µM AM85 for 40 min. CISD1 ubiquitylation (CISD1-Ub) was detected by western blotting. B) Time course of AM85 in HeLa cells. Detection of Parkin S65 phosphorylation (pS65 Parkin), Parkin, Full length and cleaved OPA1 and CISD1 ubiquitylation (CISD1-Ub) upon different time points of AM85 treatment (5, 10, 20, 40 min). C) Quantitative analysis of Serine 65 phosphorylation in response to AM85 treatment at different time points. Bars represent the average ratio ± SEM of 2 independent experiments and the value is a ratio between pSer65 Parkin and total Parkin. D) Niclosamide and AM85 uncouple mitochondria in HeLa cells. Exemplary FACS graph of CMXRos fluorescence intensity in Hela cells treated on site. Hela cells were treated on site with antimycin A and oligomycin A (A/O, green), niclosamide (Niclo, red), AM85 (blue) and the vehicle, DMSO (black).
VI. Supplementary figure 3

Supplementary 3. Mitochondrial membrane potential is restored upon wash out of AM85 and niclosamide. A) Exemplary FACS curve of CMXRos fluorescence intensity in Hela cells subjected to 30 min washout treatment. Hela cells treated with Antimycin A/ Oligomycin A (A/O, green curve), niclosamide (Niclo, red curve), AM85 (blue curve), normalized to the vehicle, DMSO (black curve). B) Niclosamide and AM85 do not induce cell death. Percentage of alive (bottom percentage) and dead cells (upper percentage) after niclosamide (bottom left square) and AM85 (bottom right square) treatment compared to DMSO (upper left square) and A/O (upper right square) treated cells. FSC-A (forward scatter area).