Potent antioxidant and Mitochondrial-protective Effects of ATH434, a Novel Therapeutic with Moderate Iron-binding Affinity

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Introduction

Iron is essential for energy metabolism, mitochondrial function, and maintaining cellular redox potential. Excess cellular labile ferrous iron generates reactive oxygen species leading to sustained oxidative stress and eventual cell death. Multiple System Atrophy (MSA), Parkinson's disease (PD) and Friedreich's ataxia (FA) and are neurodegenerative conditions characterized by regional excess brain iron and resultant oxidative stress, leading to clinical trials of iron-binding small molecules. ATH434, a small molecule drug candidate with moderate ferric iron affinity (K_d 10^{-10}), reduces excess brain iron and aggregated α synuclein, improves neuronal survival, and restores motor performance in murine PD and MSA models. ATH434 is currently in phase 2 MSA trials. Deferiprone (Dfp) is a drug with high ferric iron affinity (K_d 10^{-21}) approved for treating systemic iron-overload disorders. Dfp's high affinity enables reduction of toxically elevated organ iron but has potential for maladaptive pharmacological effects on iron stores in healthy cells. Although Dfp's efficacy in preclinical FA and PD models led to clinical testing, trials demonstrated adverse effects consistent with high ferric iron affinity-induced cellular iron depletion. Thus, iron-related treatments may require features that allow management of cytotoxic labile iron.

Previously, we presented data showing that ATH434 and ATH434-met could rescue the menadione-induced reduction in mitochondrial membrane potential (MMP) determined by TMRM staining, while the iron chelators Dfp and Dfx (Deferasirox) did not. We then investigated the efficacy of ATH434 and the other comparator compounds as mitochondrial protectants and assessed their antioxidant capacity. ATH434 demonstrated in-solution antioxidant activity in ABTS with an EC_{50} of 28.6 μM ± 4.1 μM, comparable to that of the Vitamin E analog Trolox (23.1 μM ± 3.4 μM) and to the concentration we've previously used for treatment with ATH434 (20 μM). We have extended our studies to include dose-response FRAP and ORAC assays, in which ATH434 performed similar or better than Trolox, while Dfx and Dfp had lower or no efficacy. We followed up our initial *in cellulo* studies with investigation of endpoints downstream of ROS production and saw that ATH434 could rescue the menadione-induced increase in lipid peroxidation back to control levels, equivalent to treatment with Trolox or α-tocopherol (Vitamin E). Further, ATH434 was able to supplement cellular energy production and at higher concentration was able to shift from mitochondrial to glycolytic ATP production that could limit ROS generation.

ATH434 has been further evaluated in neuronal models that replicate the altered cellular oxidative status of iron-related neurodegenerative diseases. In HT22 cell line-derived neurons, ATH434 potently protected plasma membranes from menadione-induced lipid peroxidation and protected cells in a hemin-induced ferroptosis model. Extensions of these findings are being conducted in neurons differentiated from FA patient iPSCs. ATH434 is currently being evaluated for protective effects on mitochondrial function, iron status, oxidative stress including lipid peroxidation, and expression of disease-related proteins, pathological markers that have been defined in the FA-patient derived neurons. Together, our results suggest that antioxidant activity is an important contributor to the efficacy of ATH434 in neurodegenerative disorders characterized by excess labile brain iron and oxidative stress, thus enhancing the efficacy of its moderate iron binding.

Conclusions & Future Directions

Together, these results suggest that antioxidant activity may be an important contributor to the efficacy of ATH434 in neurodegenerative disorders characterized by oxidative stress, enhancing the efficacy of its moderate iron binding. We conclude that:

- **434 vs Dfp**: 434 has potent antioxidant activity. Dfp does *not* possess antioxidant activity and does *not* protect cells from menadione-induced reductions in MMP.
- **434 vs 434-met**: 434 is more potent that 434-met. The electron transfer activity of 434 likely explains its potency in protection from menadione.
- **434 on energy metabolism:** 434 supports overall cellular energy production at low doses and shifts away from ROS-generating mitochondrial ATP production at doses used in cell-based assays.
- **FA iPSC neuronal model**: iPSC derived neurons from healthy control and FA patients demonstrated the expected iron overload and mitochondrial deficits. Future studies will investigate the protective properties of 434 in these neurons as a treatment for both iron overload and oxidative stress.
- **Ferroptosis model:** 434 partially protects cells from hemin-induced lipid peroxidation in a neuronal model of ferroptosis. Studies are ongoing to assess the efficacy of 434 under variable hemin conditions, and other aspects of ferroptosis such as GPX4 activity and regulation of iron status markers.

References

1. Finkelstein, David I., et al. "The novel compound PBT434 prevents iron mediated neurodegeneration and alpha-synuclein toxicity in multiple models of Parkinson's disease." *Acta neuropathologica communications* 5 (2017): 1-16. doi: 10.1186/s40478-017-0456-2
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Results

ATH434 antioxidant activity in HT22 neuronal model

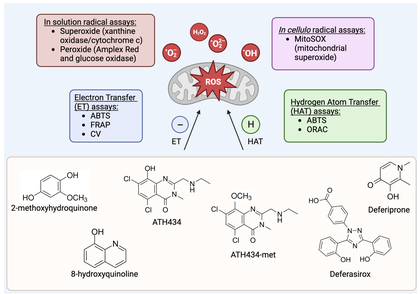
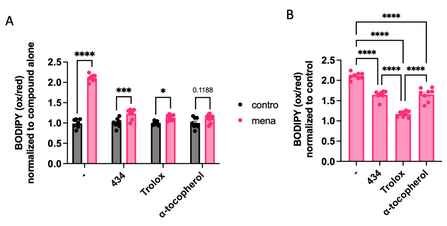
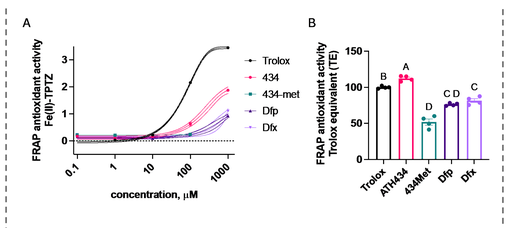


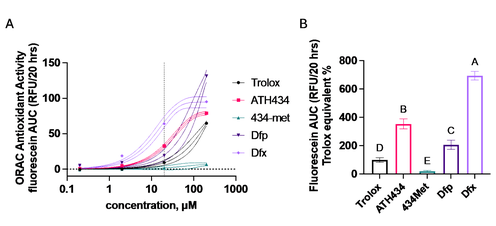
Illustration of antioxidant assays and structures of compounds used in this study.



***ATH434 pre-treatment protects HT22 neurons from menadione-induced lipid peroxidation. A**, Lipid peroxidation was determined by BODIPY assay using 10μM menadione for 20h with or without 20μM compound pre-treatment for the first 2h. **B**, Comparison of compound treatments within menadione treatment group.*



***ATH434 has electron transfer capacity. A**, FRAP assay was used to determine the ET capacity of 434, 434-met, Dfp, and Dfx in comparison to Trolox. **B**, The Trolox equivalent (TE) activity was calculated for each compound at 10μM.*



***ATH434 has hydrogen atom transfer activity. A**, ORAC assay was used to determine the HAT capacity 434, 434-met, Dfp, and Dfx in comparison to Trolox. **B**, The Trolox equivalent (TE) activity was calculated for each compound at 20μM.*

***ABTS antioxidant activity.** EC50 values were calculated for each compound based on reduction of ABTS radical. Data originally presented at SfN 2023.*

Compound	EC_{50} (μM) ± SEM	p value, compared to 434
434	28.6 ± 4.1	-
434-met	160.4 ± 12.1	****, p < 0.0001
Dfp	not calculable	n.d.
Dfx	197.9 ± 35.8	****, p < 0.0001
Trolox	23.1 ± 3.4	ns, p = 0.9998
8-quinolinol	4.1 ± 0.4	ns, p = 0.9385
2-methoxyhydroquinone	387.3 ± 39.4	****, p < 0.0001

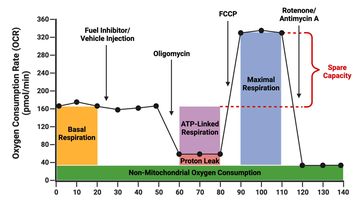
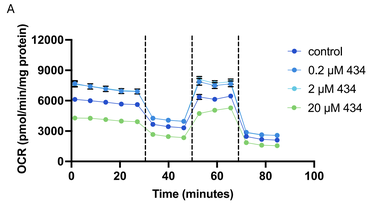


***Diagram of Seahorse metabolic assay and output parameters.** From Mayberry, C.L., et al. STAR protocols (2024).*

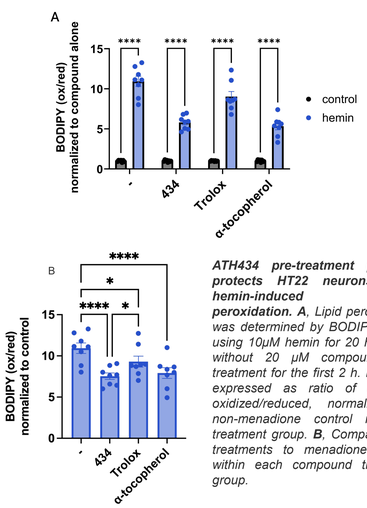


***Seahorse metabolic analysis of HT22 cells pre-treated with increasing concentrations of ATH434. A,** Oxygen consumption rate in cells pre-treated with ATH434. **B,** Contributions of glycolysis and mitochondrial oxidative phosphorylation to ATP production.*

Seahorse parameter	control	0.2μM 434	2μM 434	20μM 434
basal glycolysis (pmol H^+/min/mg protein)	5317 ± 1011	6988 ± 1516	7539 ± 1591	8260 ± 1509
basal ox phos (pmol O_2/min/mg protein)	6125 ± 912	7686 ± 1410	7604 ± 1226	4275 ± 726
glycolytic ATP (pmol/min/mg protein)	6709 ± 1141	8769 ± 1808	9341 ± 1747	9223 ± 1688
mitochondrial ATP (pmol/min/mg protein)	12660 ± 1634	16222 ± 3264	16376 ± 2816	8704 ± 1837
ATP-linked respiration (pmol O_2/min/mg protein)	2302 ± 297	2949 ± 594	2977 ± 512	1583 ± 334
proton leak (pmol O_2/min/mg protein)	1216 ± 243	1391 ± 340	1377 ± 249	784 ± 154

ATH434 in other oxidative stress models

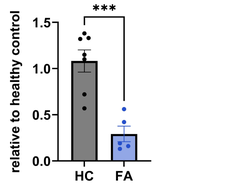
ATH434 efficacy in hemin-induced ferroptosis model



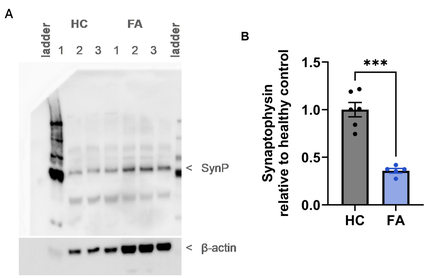
***ATH434 pre-treatment partially protects HT22 neurons from hemin-induced lipid peroxidation. A**, Lipid peroxidation was determined by BODIPY assay using 10μM hemin for 20 h with or without 20 μM compound pre-treatment for the first 2 h. Data are expressed as ratio of BODIPY oxidized/reduced, normalized to non-menadione control in each treatment group. **B**, Comparison of treatments to menadione control within each compound treatment group.*

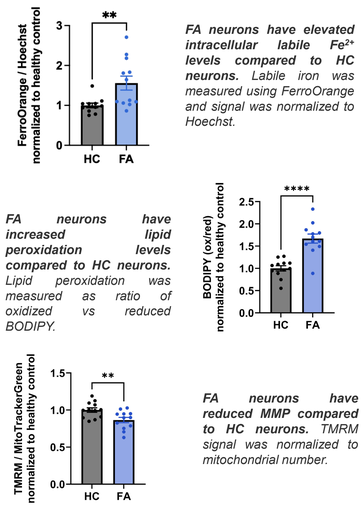
Characterization of iPSC-derived Friedreich's ataxia (FA) model

***FA-patient iPSC-derived neurons have decreased FXN levels compared to healthy controls.** FXN levels were determined by qPCR analysis and normalized to β-actin housekeeping gene.*



***FA neurons have decreased expression of MAP2, β-III-tubulin, and Synaptophysin (below). A,** Western blots of Synaptophysin and β-actin protein. **B,** Quantification of Synaptophysin normalized to β-actin loading control, and expressed relative to HC.*

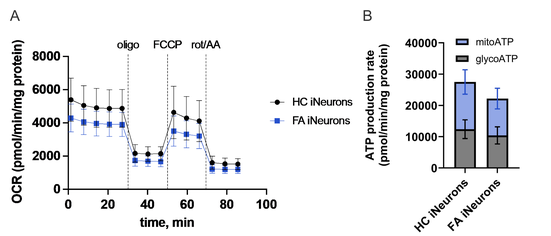




***FA neurons have elevated intracellular labile Fe^{2+} levels compared to HC neurons.** Labile iron was measured using FerroOrange and signal was normalized to Hoechst.*

***FA neurons have increased lipid peroxidation levels compared to HC neurons.** Lipid peroxidation was measured as ratio of oxidized vs reduced BODIPY.*

***FA neurons have reduced MMP compared to HC neurons.** TMRM signal was normalized to mitochondrial number.*



Seahorse parameter	HC	FA
basal glycolysis (pmol H^+/min/mg protein)	11190 ± 2572	9049 ± 1594
basal ox phos (pmol O_2/min/mg protein)	5392 ± 1298	4182 ± 972
glycolytic ATP (pmol/min/mg protein)	12391 ± 2984	10408 ± 2725
mitochondrial ATP (pmol/min/mg protein)	15103 ± 3912	12278 ± 2288

***FA neurons have reduced glycolytic and oxidative metabolism compared to HC neurons. A**, Oxygen consumption (OCR) trace was reported. **B**, Total ATP production from glycolysis and mitochondrial oxidative phosphorylation.*

Calculated values for Seahorse parameters are reported in table (left).

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