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# Insights into the role of oxidative stress in the pathology of Friedreich ataxia using peroxidation resistant polyunsaturated fatty acids



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## ABSTRACT

Friedreich ataxia is an autosomal recessive, inherited neuro- and cardio-degenerative disorder characterized by progressive ataxia of all four limbs, dysarthria, areflexia, sensory loss, skeletal deformities, and hypertrophic cardiomyopathy. Most disease alleles have a trinucleotide repeat expansion in the first intron of the *FXN* gene, which decreases expression of the encoded protein frataxin. Frataxin is involved in iron–sulfur-cluster (ISC) assembly in the mitochondrial matrix, and decreased frataxin is associated with ISC-enzyme and mitochondrial dysfunction, mitochondrial iron accumulation, and increased oxidative stress. To assess the role of oxidative stress in lipid peroxidation in Friedreich ataxia we used the novel approach of treating Friedreich ataxia cell models with polyunsaturated fatty acids (PUFAs) deuterated at bis-allylic sites. In ROS-driven oxidation of PUFAs, the rate-limiting step is hydrogen abstraction from a bis-allylic site; isotopic reinforcement (deuteration) of bis-allylic sites slows down their peroxidation. We show that linoleic and  $\alpha$ -linolenic acids deuterated at the peroxidation-prone bis-allylic positions actively rescue oxidative-stress-challenged Friedreich ataxia cells. The protective effect of the deuterated PUFAs is additive in our models with the protective effect of the CoQ10 analog idebenone, which is thought to decrease the production of free radicals. Moreover, the administration of deuterated PUFAs resulted in decreased lipid peroxidation as measured by the fluorescence of the fatty acid analog C11-BODIPY (581/591) probe. Our results are consistent with a role for lipid peroxidation in Friedreich ataxia pathology, and suggest that the novel approach of oral delivery of isotope-reinforced PUFAs may have therapeutic potential in Friedreich ataxia and other disorders involving oxidative stress and lipid peroxidation.

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## Introduction

The clinical presentation, molecular genetics, and pathogenesis of Friedreich ataxia are the subjects of several excellent recent reviews [1–5]. Briefly, Friedreich ataxia is the most common hereditary ataxia, with a prevalence of approximately 1 in 50,000

*Abbreviations:* BSO, buthionine (S,R)-sulfoximine; CNS, central nervous system; D-, deuterated; DMEM, Dulbecco's modified Eagle's medium; FAC, ferric ammonium citrate; FBS, fetal bovine serum; ISC, iron–sulfur cluster; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; YPD, yeast-extract peptone dextrose.

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in European populations. The neurological signs and symptoms, comprising ataxia, dysarthria, areflexia, and sensory loss, are largely secondary to degeneration of the large sensory neurons of the dorsal root ganglia and spinocerebellar tracts. Onset of symptoms usually occurs around puberty and most patients are confined to a wheelchair by their late 20s. Myocardial failure and/or arrhythmias are the most common cause of premature death. Currently, there are no approved drugs to treat Friedreich ataxia and the resultant disability, prolong the life of a Friedreich ataxia patient, or cure the disorder.

The Friedreich ataxia disease protein, frataxin, localizes to the mitochondrial matrix where it binds iron and is involved in iron–sulfur-cluster (ISC) assembly [6,7]. ISCs are prosthetic groups important for the function of many proteins, both mitochondrial and cytosolic, including aconitase and mitochondrial respiratory complexes I, II, and III. The decrease in frataxin function in Friedreich ataxia results in decreased ISC assembly; in Friedreich ataxia patients, as well as in yeast and mouse models, this is associated with mitochondrial dysfunction, mitochondrial iron accumulation, cytosolic iron depletion, and increased oxidative stress [8–22].

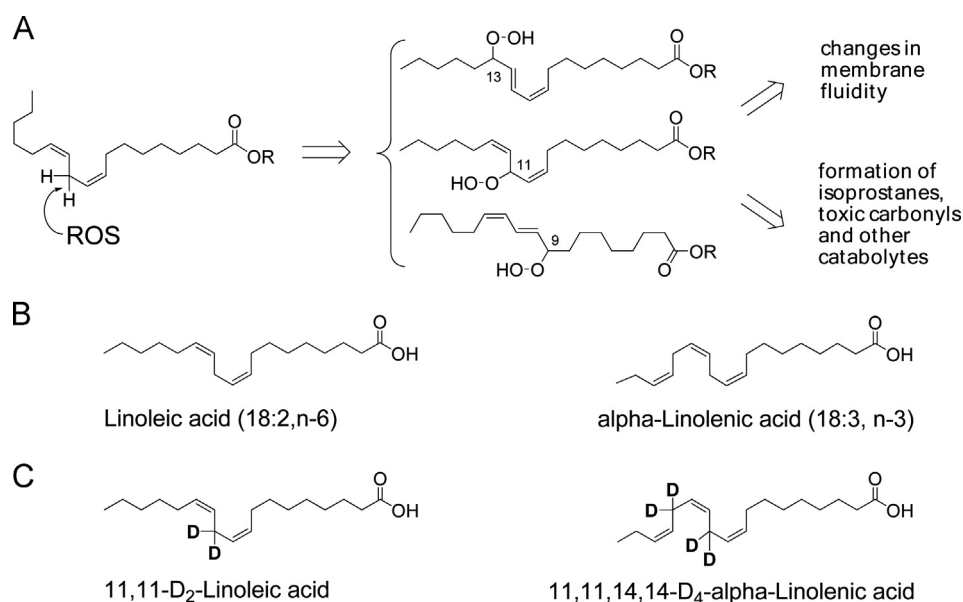
The evidence for oxidative stress in Friedreich ataxia is the subject of an excellent recent review by Armstrong et al. [23] That there should be oxidative stress in Friedreich ataxia, and that it might be difficult to measure directly, is unsurprising. The iron that accumulates in the mitochondria of cells with decreased frataxin function is primarily ferrous. Ferrous iron can generate toxic reactive oxygen species (ROS) through Fenton and related Haber–Weiss reactions [24], initiating detrimental processes such as lipid peroxidation [25,26]. The mitochondrial inner membrane is particularly susceptible to lipid peroxidation due to the high content of polyunsaturated fatty acids (discussed in detail below), and lipid peroxidation is thought to play an important role in neurodegenerative disease generally [27]. However, a certain level of physiologic ROS is present normally, which can obscure low-level signals from a relatively small number of affected disease cells (such as in Friedreich ataxia), and cells with extremes of oxidative stress often die by apoptosis. The preponderance of evidence, both direct and indirect, supports the hypothesis that oxidative stress contributes to Friedreich ataxia pathophysiology.

Nevertheless, the success of antioxidant-based therapies has been limited, for Friedreich ataxia and for other disorders, for several possible reasons, including lack of penetration across the blood-brain barrier, the near-saturating amount of antioxidants already present in living cells, the importance of ROS in cell signaling, hormetic up-regulation of protective mechanisms, and the potential toxicities of some antioxidants [28–36]. For example, tocopherols (vitamin E) can have both anti-oxidant and pro-oxidant effects [28]; such dual effects can depend on whether physiologic or supra-physiologic levels are achieved [36]. Idebenone, a short-chain parabenzoquinone derivative with a structure very similar to that of Coenzyme Q10 (CoQ10), advanced to phase III trials for Friedreich ataxia. Like CoQ10, idebenone transfers electrons from complex I and complex II to complex III in the mitochondrial electron transport chain [37]. Both open trials, and small double-blind placebo-controlled trials, indicated some indirect evidence for efficacy of idebenone for the cardiomyopathy of Friedreich ataxia, but little evidence for efficacy for the ataxia of Friedreich ataxia [30–32,34]. In phase III trials of idebenone, and in open-label extension studies, there were trends toward

improvement in ataxia-scale scores and cardiac hypertrophy, but statistical significance was not achieved [38–40].

Polyunsaturated fatty acids (PUFAs) such as linoleic acid (C18:2, *n*-6) and  $\alpha$ -linolenic acid (C18:3, *n*-3) are essential fatty acids [41] from which all higher PUFAs can be built [42,43]. CNS tissues are rich in PUFAs [44]; the mitochondrial phospholipid cardiolipin, which contains linoleic acid, is vital for mitochondrial function and, upon oxidation, plays a key role in apoptosis [45]. In addition to their vital role in lipid membranes, PUFAs are easy targets for ROS in Friedreich ataxia due to the vicinity of ROS formation (at the ISC-containing mitochondrial membrane proteins) to the PUFA-rich mitochondrial membranes. PUFA autoxidation is a chain reaction initiated by ROS whereby a small number of ROS can damage a large number of PUFA molecules before the process is terminated [46], changing the membrane fluidity [47] and yielding a wide array of lipid autoxidation products, such as toxic  $\alpha,\beta$ -unsaturated carbonyls like malondialdehyde, 4-hydroxynonenal, and acrolein [48], which cannot be neutralized by simple chain-termination strategies (Fig. 1A).

The excess mitochondrial iron associated with Friedreich ataxia can catalyze the generation of hydroxyl radicals, which are so reactive that they cause oxidative damage, such as initiation of PUFA autoxidation, at their diffusion rate. Once lipid peroxides are formed, iron can further accelerate their decomposition, which, followed by  $\beta$  scission, gives rise to the whole smorgasbord of toxic carbonyls, as described above [48]. For the reasons specified above, in addition to a particularly high activity of the hydroxyl radical, which is a function of its diffusion rate, conventional antioxidants are not viable as a counteracting strategy and novel approaches to the inhibition of lipid peroxidation processes are required. It is in this context that we have proposed a novel lipid isotope stabilization strategy, which employs the isotope effect to inhibit lipid peroxidation [49–51]. In ROS-driven oxidation of PUFAs, the rate-limiting step is hydrogen abstraction from a bis-allylic site; isotopic reinforcement (deuteration) of bis-allylic sites, while not changing the chemical nature of linoleic acid and  $\alpha$ -linolenic acid, slows down their peroxidation (Fig. 1B). Isotopically reinforced linoleic acid and  $\alpha$ -linolenic acid (Fig. 1C) provide a tool to further assess the hypothesis that oxidative stress, particularly



**Fig. 1.** (A) ROS-driven oxidation of PUFAs. The rate-limiting step is ROS-driven hydrogen abstraction from a bis-allylic site. The end products of peroxidation are reactive carbonyl compounds such as malondialdehyde and 4-hydroxynonenal, which irreversibly damage proteins and DNA by forming cross-links. (B) Linoleic acid and  $\alpha$ -linolenic acid are the *n*-6 and *n*-3 essential PUFAs from which all higher PUFAs can be built. CNS tissues are rich in PUFAs; the mitochondrial phospholipid cardiolipin, which contains linoleic acid, is vital for mitochondrial function and, upon oxidation, plays a key role in apoptosis. (C) Isotope protection (deuteration) of bis-allylic sites, while not changing the chemical nature of linoleic acid (D<sub>2</sub>-Lin) and  $\alpha$ -linolenic acid (D<sub>2</sub>-Lnn), slows down their peroxidation.

lipid peroxidation, contributes to Friedreich ataxia pathophysiology, and to preliminarily assess whether dietary supplementation with isotope-reinforced PUFAs might have therapeutic potential in Friedreich ataxia. Thus, we sought to determine whether isotopically reinforced PUFAs are protective in oxidative-stress-challenged Friedreich ataxia cells.

## Materials and methods

### Deuterated poly-unsaturated fatty acids (D-PUFAs)

11, 11-D<sub>2</sub>-linoleic acid and 11,11,14,14-D<sub>4</sub>- $\alpha$ -linolenic acid were prepared as described previously [49] and used as free acids. Non-deuterated PUFAs were obtained from Sigma-Aldrich (99%; St. Louis, MO, USA).

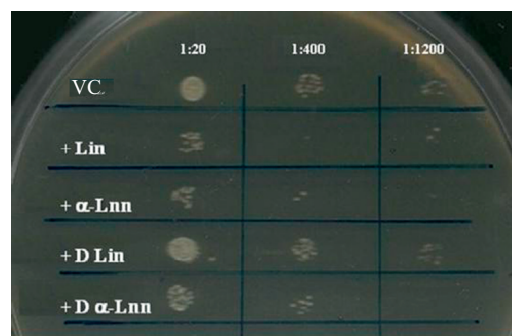
### Friedreich ataxia cell models

Frataxin and Yfh1p, the yeast frataxin homolog, are functional homologs [9], and virtually every finding in yeast models of Friedreich ataxia has been confirmed in conditional knockout mice, primary human fibroblasts, and patients with Friedreich ataxia. The yeast model of Friedreich ataxia used in this study lacks the *Yfh1* gene completely ( $\Delta yfh1::HIS3$ ) and is derived from the wild-type strain BY4741 (MATA *leu2 $\Delta$ 0 met15 $\Delta$ 0 ura3 $\Delta$ 0 his3 $\Delta$ 1*). Yeast were grown in standard, Yeast-extract Peptone Dextrose (YPD), medium. To test the effects of PUFAs and D-PUFAs on yeast, cells were grown to early log phase, washed twice with water, resuspended in 0.1 M phosphate buffer at pH 6 supplemented with 0.2% glucose, and incubated in the presence of vehicle control (ethanol) or PUFAs at a final concentration of 200  $\mu$ M for 4 h. The cells were then resuspended in medium, allowed to grow overnight, and spotted on solid medium at dilutions of 1:20, 1:400, and 1:1200. To detect lipid peroxidation, yeast cells were grown and incubated with H- or D-PUFAs for 2 h as described above. Cells were then treated with C11-BODIPY (Molecular Probes, Eugene, OR, USA) at 8  $\mu$ M final concentration for 30 min at room temperature. Fluorescence was measured on a BioTek plate reader using a 485/20 excitation filter and a 520/20 emission filter and normalized for the number of cells (optical density at 630 nm) [52,53].

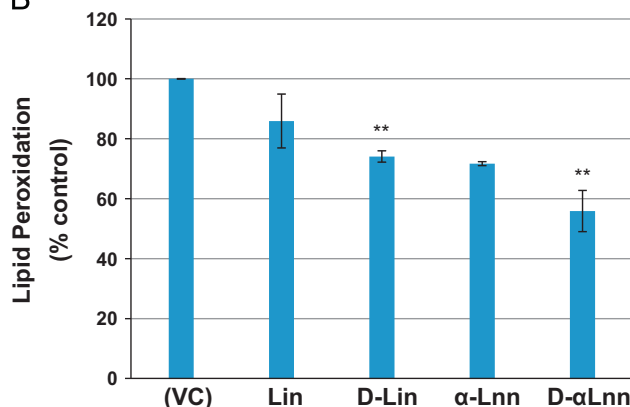
The murine Friedreich ataxia cell model used in this study was developed by Puccio and colleagues [54], and is a fibroblast cell line in which both alleles encoding murine frataxin are deleted and replaced by human frataxin harboring the disease-associated missense mutation, I154F. This model exhibits virtually all of the biochemical hallmarks of Friedreich ataxia, including mitochondrial iron accumulation, loss of iron-sulfur-cluster-enzyme activities, and sensitivity to iron and oxidative stress. The human Friedreich ataxia cells used in this study are primary human Friedreich ataxia fibroblasts (Coriell designation GM3665) with GAA repeat expansions of 790 and 1357 repeats in the first intron of the Friedreich ataxia disease gene. Relative to control cells (murine fibroblasts in which murine frataxin is replaced by wild-type human frataxin; primary human fibroblasts), both mammalian Friedreich ataxia models show increased sensitivity to treatment with iron, as ferric ammonium citrate (FAC), and L-buthionine (S,R)-sulfoximine (BSO), which inhibits the rate-limiting step of glutathione synthesis. We have established conditions under which concentrations of iron and BSO, that by themselves are non-toxic, together cause a synergistic loss of viability [55]. In addition, in validation experiments using drugs currently in clinical trials for Friedreich ataxia, we have shown that these assays have excellent sensitivity and dynamic range, and allow a precise quantitative assessment of dose-response [55,56]. Briefly, cells were grown in standard Dulbecco's modified Eagle's

medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. For the murine fibroblasts, 12,000 cells/well were seeded into 48-well microtiter plates and FAC (F-5879, Sigma, St. Louis, MO, iron content 16.5–18.5%) was added to a final concentration of 20  $\mu$ g/mL (Day 0). The following day (Day 1), BSO was added to a 50  $\mu$ M final concentration, and 2 h later compounds (or vehicle alone) were added. Forty-eight hours later (Day 4), the cells were washed with phosphate-buffered saline (PBS) and viability was assessed by chemiluminescence measurement of intracellular ATP using the CellTiter-Glo assay kit (Promega, Madison, WI) as per the manufacturer's instructions. The human fibroblasts were tested similarly, except they were seeded at 3000 cells/well in 96-well plates, compounds were added 2 h after the addition of FAC, BSO was added on Day 2, and viability was quantified on Day 3. To detect lipid peroxidation, cells were treated with FAC and BSO as described above, washed with PBS and incubated with C11-BODIPY 581/591 at a final concentration of 8  $\mu$ M for 3 h at 37 °C. Fluorescence was measured on a BioTek plate reader using a 485/20 excitation filter and a 520/20 emission filter. Cell viability was assessed in parallel lanes as described above and used to normalize the fluorescence values.

A



B



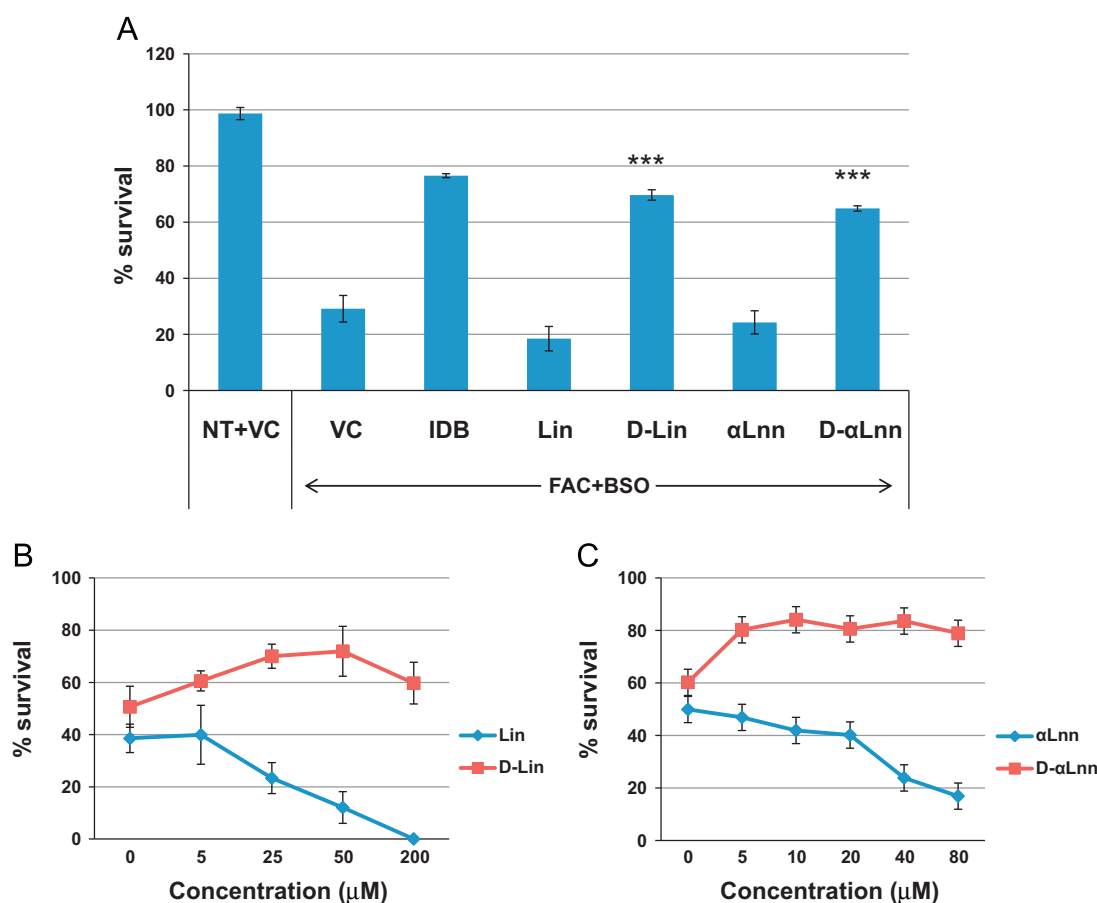
**Fig. 2.** (A) In yeast lacking *Yfh1p*, deuteration of PUFAs alleviates toxicity. Cells were incubated in the presence of vehicle control (VC), linoleic acid (Lin),  $\alpha$ -linolenic acid ( $\alpha$ -Lnn), or their corresponding deuterated forms (D-Lin, D- $\alpha$ -Lnn); grown overnight, and plated at three different dilutions (1:20, 1:400, 1:1200). Yeast cells incubated in the presence of linoleic acid or  $\alpha$ -linolenic acid exhibited significantly less growth compared to cells incubated with their corresponding deuterated forms (D-Lin, D- $\alpha$ -Lnn; row 2 vs. row 4, and row 3 vs. row 5, respectively). (B) In yeast lacking *Yfh1p*, deuteration of PUFAs decreases lipid peroxidation. Cells were incubated for 2 h in the presence of vehicle control (VC), linoleic acid (Lin),  $\alpha$ -linolenic acid ( $\alpha$ -Lnn), or their corresponding deuterated forms (D-Lin, D- $\alpha$ -Lnn) and then treated with C11-BODIPY (581/591) dye at 8  $\mu$ M for 30 min at room temperature. Fluorescence was measured on a plate reader using 485/20 (excitation) and 520/20 (emission) filters and normalized by cell density (optical density at 630 nm). Data are represented as the percentage of lipid peroxidation relative to vehicle control (VC). \*\*,  $p < 0.005$  by two-sided *t*-test. The data shown are the mean and the standard deviation calculated from three independent replicates.

## Results

We first tested whether a yeast strain lacking the *Yfh1* gene is susceptible to PUFA-induced loss of viability and whether deuteration of the PUFAs can reverse this effect. Yeast cells lacking *Yfh1*, and the corresponding wild-type strain, were incubated in the presence of vehicle control (ethanol), linoleic acid,  $\alpha$ -linolenic acid, or their corresponding deuterated forms (D-linoleic acid or D- $\alpha$ -linolenic acid, respectively), at a final concentration of 200  $\mu$ M for 4 h (Section Materials and methods). The cells were then resuspended in medium, allowed to grow overnight, and spotted on solid medium at three different dilutions. Yeast cells incubated in the presence of linoleic acid or  $\alpha$ -linolenic acid exhibited significantly less growth compared to cells incubated with D-linoleic acid or D- $\alpha$ -linolenic acid, whereas there was no effect on the viability of the wild-type strain used as a control (Fig. 2A). To test whether treatment with PUFAs also affects lipid peroxidation, yeast cells lacking *Yfh1* were incubated with H- or D-PUFAs for 2 h and then treated with the fluorescent fatty acid analog C11-BODIPY, a sensitive indicator of lipid peroxidation and antioxidant efficacy in membrane systems and living cells. Oxidation of C11-BODIPY (581/591) induces a fluorescence shift from red to green [57]. Although the fluorescence does not yield strictly quantitative information about lipid peroxidation, it does provide

a sensitive readout of radical processes that oxidize lipids in membranes [58]. Treatment with D-PUFAs resulted in 25% and 45% decreased lipid peroxidation for D-linoleic acid and D- $\alpha$ -linolenic acid, respectively (Fig. 2B). Treatment with H- $\alpha$ -linolenic acid also resulted in a statistically significant reduction of lipid peroxidation, reflecting perhaps a non-specific benefit that the yeast cells gain by having a poly-unsaturated fatty acid in their membranes. D-PUFAs also decreased lipid peroxidation in wild-type yeast cells that were oxidatively stressed with 50  $\mu$ M CuSO<sub>4</sub> (data not shown).

We then tested the effects of H- and D-PUFAs using the murine fibroblast, I154F missense-mutation model of Friedreich ataxia. These cells are sensitive to the combination of iron (as FAC) and BSO (which inhibits glutathione synthesis) at concentrations that by themselves are non-toxic but together cause a synergistic loss of viability. In Fig. 3A, we show that both D-linoleic acid and D- $\alpha$ -linolenic acid rescue these cells from loss of viability at 16  $\mu$ M ( $p < 0.001$ ) similarly to 1  $\mu$ M idebenone, the CoQ10 analog tested in drug trials for Friedreich ataxia. In Fig. 3B we show that as concentrations were increased, H-linoleic acid became more and more toxic in the presence of FAC and BSO, whereas D-linoleic acid continued to rescue at 50  $\mu$ M. Likewise, in Fig. 3C, we show that as concentrations were increased, H- $\alpha$ -linolenic acid became more and more toxic in the presence of FAC and BSO, whereas D- $\alpha$ -



**Fig. 3.** D-PUFAs are protective in the murine fibroblast, I154F missense-mutation model of Friedreich ataxia, whereas H-PUFAs are toxic. Cells were seeded in 48-well plates and treated with a combination of FAC (ferric ammonium citrate) and BSO (L-buthionine (S,R)-sulfoximine) for 72 h and 48 h, respectively; PUFAs were added 2 h after BSO addition; and viability was assessed by chemiluminescence measurement of intracellular ATP (Materials and Methods). The data represent the percent survival relative to untreated control cells. The final concentration of the drug vehicle (ethanol) was 0.5%. The data shown are the mean and the standard deviation calculated from six independent replicates. (A) H-linoleic acid, H- $\alpha$ -linolenic acid, D-linoleic acid, and D- $\alpha$ -linolenic acid were tested at 16  $\mu$ M, and idebenone (IDB) was tested at 1  $\mu$ M. NT, Not Treated (with FAC and BSO); VC, Vehicle Control; \*\*\*,  $p < 0.001$  by two-sided *t*-test. (B) Dose response with H-linoleic acid and D-linoleic acid, with survival normalized to the viability of the cells treated with vehicle control only. (C) Dose response with H- $\alpha$ -linolenic acid and D- $\alpha$ -linolenic acid, with survival normalized to the viability of the cells treated with vehicle control only.

linolenic acid continued to rescue at 80  $\mu\text{M}$ . Using the same model, we tested whether the effects of the D-PUFAs and idebenone were additive, which would be consistent with different mechanisms of action. Fig. 4 shows that at concentrations of D-linoleic acid and D- $\alpha$ -linolenic acid that were only marginally protective (1.6  $\mu\text{M}$ ), the addition of idebenone, also at a marginally effective concentration (100 nM), resulted in an additive protective effect.

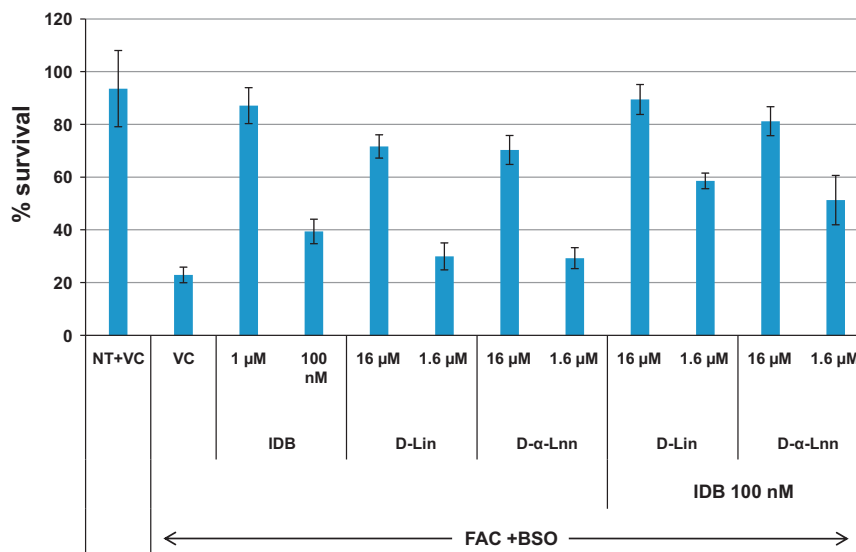
We then tested the effects of H- and D-PUFAs using primary human Friedreich ataxia fibroblasts. These cells are also sensitive to the combination of iron (as FAC) and BSO (which inhibits glutathione synthesis) at concentrations that by themselves are non-toxic but together cause a synergistic loss of viability. In Fig. 5A, we show that both D-linoleic acid and D- $\alpha$ -linolenic acid rescue these cells from loss of viability at 10  $\mu\text{M}$  ( $p < 0.001$ ), similarly to 1  $\mu\text{M}$  idebenone. In Fig. 5B we show that as concentrations were increased, H-linoleic acid became more and more toxic in the presence of FAC and BSO, whereas D-linoleic acid continued to rescue at 80  $\mu\text{M}$ . Likewise, in Fig. 5C, we show that as concentrations were increased, H- $\alpha$ -linolenic acid became more and more toxic in the presence of FAC and BSO, whereas D- $\alpha$ -linolenic acid continued to rescue at 80  $\mu\text{M}$ .

Lipid peroxidation in the membranes of primary human Friedreich ataxia fibroblasts (Coriell designation GM3665) was monitored using C11-BODIPY (581/591). The fibroblasts were stressed with iron plus BSO and treated with PUFAs as described above and then incubated with the C11-BODIPY. Lipid peroxidation in the cells treated with FAC and BSO increased 2.5 fold relative to cells treated with vehicle control alone and was unchanged by the addition of H-PUFAs. In contrast, treatment with D-PUFAs resulted in significantly lower levels of lipid peroxidation (Fig. 6). We also tested the effects of H- and D-PUFAs on both the mouse and human FA-cell models in the absence of any exogenous oxidative stress; neither the H-PUFAs nor the D-PUFAs affected cell viability up to 80  $\mu\text{M}$  and 72 h of treatment (data not shown).

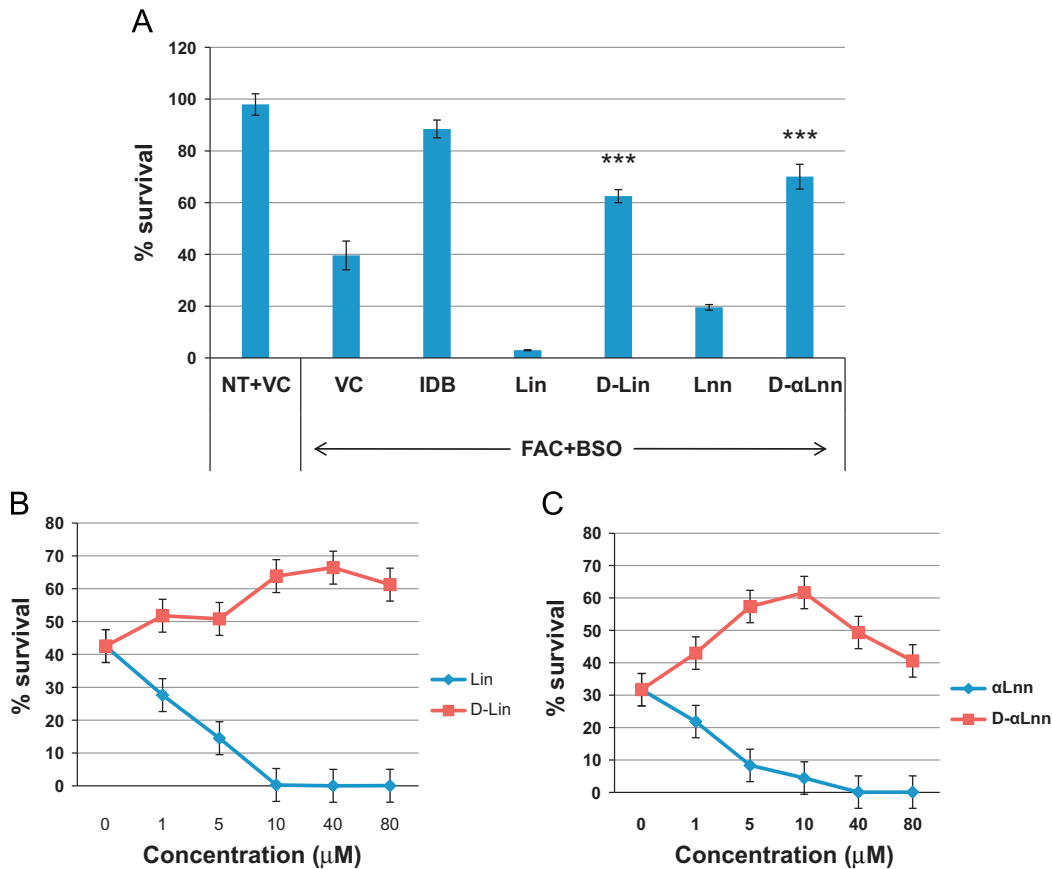
## Discussion

In our yeast model of Friedreich ataxia, the PUFAs linoleic acid and  $\alpha$ -linolenic acid were toxic in the micromolar range, and deuteration of these PUFAs at bis-allylic sites eliminated the toxicity. Our mammalian-cell models of Friedreich ataxia are particularly sensitive to oxidative stress with FAC and BSO; in both of these models, H-linoleic acid and H- $\alpha$ -linolenic acid in the micromolar range significantly exacerbated the toxicity of FAC and BSO, whereas their deuterated counterparts, D-linoleic acid and D- $\alpha$ -linolenic acid, were actively protective at the same concentrations. H-linoleic acid and H- $\alpha$ -linolenic acid are known substrates for PUFA-oxidation chain reactions initiated by ROS, so their toxicity in this context is not surprising. We expected that replacement of bis-allylic hydrogen atoms in linoleic acid and  $\alpha$ -linolenic acid with the heavier stable isotope, deuterium (D), would stabilize the C-H bond that is first broken in lipid peroxidation, thereby decreasing the formation of intracellular toxic lipid peroxides and diminishing the activation of toxic cellular cascades. Our results in Friedreich ataxia models are consistent with this expectation, as well as with results in other disease models in which oxidative stress is thought to play an important role [49–51].

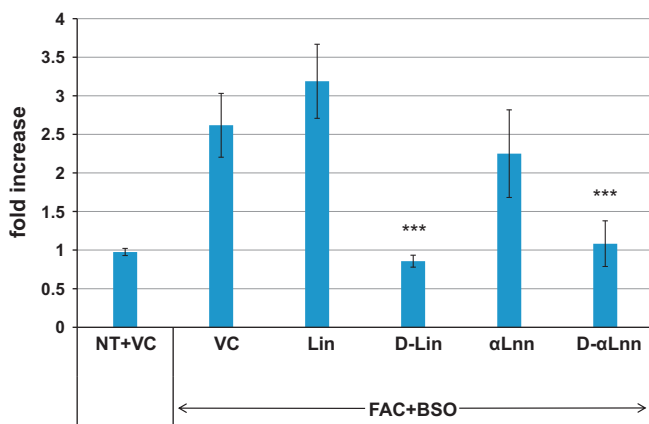
The significance of our results is threefold: first, while oxidative stress in Friedreich ataxia has been difficult to measure, the protective effects of D-PUFAs in all three of our Friedreich ataxia cell models indicate, directly in the yeast cells and indirectly in the mammalian cells, that there is oxidative stress in these models and emphasize the importance of lipid peroxidation. Second, our results further support the proposal that reinforcing essential PUFAs with isotopic replacement decreases lipid peroxidation and decelerates toxic cellular cascades [59]. Third, together with the results of Hill et al., who showed that substitution of as little as 20% of PUFAs with appropriately deuterated forms can be



**Fig. 4.** Additive effects of D-PUFAs plus idebenone on I154F cells treated with FAC+BSO. Murine fibroblast cells were seeded in 48-well plates and treated with FAC and BSO as described in Fig. 3A. Two hours after the addition of BSO, the cells were treated with idebenone (IDB; columns 3, 4), D-linoleic acid (D-Lin; columns 5, 6), or D- $\alpha$ -linolenic acid (D- $\alpha$ -Lnn; columns 7, 8) at two different concentrations, or a combination of idebenone and D-linoleic acid (columns 9, 10) or D- $\alpha$ -linolenic acid (columns 11, 12), at the concentrations indicated. After 2 more days, cells were lysed and their viability assessed by chemiluminescence measurement of intracellular ATP. The data represent the percent survival relative to untreated control cells. Drug vehicle (VC, ethanol) final concentration was 0.5%. Idebenone (columns 3 and 4) was efficacious at both concentration used (two sided  $p$  value  $p < 0.001$ , column 4 vs. column 2 cells treated with FAC+BSO and no drug). D-linoleic acid and D- $\alpha$ -linolenic acid were able to rescue the cells only when used at 16  $\mu\text{M}$  but not when used at 1.6  $\mu\text{M}$  (columns 5 and 7 vs. 6 and 8, respectively). The combination of idebenone at 100 nM plus D-linoleic acid at 1.6  $\mu\text{M}$  increased cell survival compared to cells treated with either idebenone or D-linoleic acid alone (column 10 vs. column 4 or column 6; two-sided  $p < 0.001$  in both cases). The combination of idebenone at 100 nM plus D- $\alpha$ -linolenic acid at 1.6  $\mu\text{M}$  increased cell survival compared to cells treated with either idebenone or with D- $\alpha$ -linolenic acid alone (column 12 vs. column 4 or column 8; two-sided  $p$  values 0.001 and 0.0005, respectively). The means and standard deviations were calculated from six independent replicates.



**Fig. 5.** D-PUFAs are protective in primary human Friedreich ataxia fibroblasts oxidatively stressed with FAC and BSO, whereas H-PUFAs are toxic. Cells were seeded in 96-well plates and treated with a combination of FAC (ferric ammonium citrate) and BSO (L-buthionine (S,R)-sulfoximine) for 96 h and 48 h, respectively; PUFAs were added two hours after FAC addition; and viability was assessed by chemiluminescence measurement of intracellular ATP (Section Materials and methods). The data represent the percent survival relative to untreated control cells. The final concentration of the drug vehicle (ethanol) was 0.5%. The data shown are the mean and the standard deviation calculated from six independent replicates. (A) H-linoleic acid, H- $\alpha$ -linolenic acid, D-linoleic acid, and D- $\alpha$ -linolenic acid were tested at 10  $\mu$ M, and idebenone (IDB) was tested at 1  $\mu$ M. NT, Not Treated (with FAC and BSO); VC, Vehicle Control; \*\*\*,  $p < 0.001$  by two-sided  $t$ -test. (B) Dose response with H-linoleic acid and D-linoleic acid, with survival normalized to the viability of the cells treated with vehicle control only. (C) Dose response with H- $\alpha$ -linolenic acid and D- $\alpha$ -linolenic acid, with survival normalized to the viability of the cells treated with vehicle control only.



**Fig. 6.** D-PUFAs decrease lipid peroxidation in primary human Friedreich ataxia fibroblasts oxidatively stressed with FAC and BSO. Cells were seeded in 96-well plates and treated as described in Fig. 5. Forty eight hours after the addition of BSO, the cells were washed and treated with C11-BODIPY dye at 8  $\mu$ M final concentration. In parallel lanes, cell viability was assessed by chemiluminescence measurement of intracellular ATP (Section Materials and methods) and used to normalize the fluorescence values. The data represent the fold increase in fluorescence relative to untreated control cells. The data shown are the mean and the standard deviation calculated from three independent replicates. The PUFAs were tested at 16  $\mu$ M. Lin, H-linoleic acid;  $\alpha$ -Lnn, H- $\alpha$ -linolenic acid; D-Lin, D-linoleic acid; D- $\alpha$ -Lnn, D- $\alpha$ -linolenic acid; NT, Not Treated (with FAC and BSO); VC, Vehicle Control; \*\*\*,  $p < 0.001$  by two-sided  $t$ -test.

protective [51], our results suggest that oral delivery D-PUFAs might be an effective therapeutic strategy for the treatment of Friedreich ataxia. A limitation of the present study is that the primary intracellular location where D-PUFAs exert their protective effects in our models remains undefined; however, given the deleterious effects of frataxin mutations on the mitochondrial electron transport chain, and the presence of mitochondrial iron accumulation in Friedreich ataxia, it seems likely that the PUFA-rich inner mitochondrial membranes are the primary site.

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