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Friedreich's ataxia (FRDA) is the most common form of inherited ataxia in the world, affecting roughly 1:50,000 people in the United States. It is inherited in an autosomal recessive manner due to a GAA trinucleotide repeat expansion in the first intron of the FXN gene on chromosome 9q13-21, causing gene silencing and a functional absence of the mitochondrial-localizing protein frataxin. The frataxin protein is responsible for the assembly of iron-sulfur centers in mitochondrial proteins, including the electron transport chain complex I-III, heme synthesis, as well as removing iron from around the mitochondria, preventing the formation of reactive oxygen species (ROS). The loss of FXN function causes an accumulation of mitochondrial iron and ROS, as well as impaired function of Fe-S centers in mitochondrial proteins, leading to mitochondrial damage and a decrease in activity of mitochondrial complexes I-III. The damaged mitochondria are unable to match ATP production to the cell's energy requirements, resulting in cell death. High energy use cell types, such as neurons and cardiac myocytes, depend almost entirely on oxidative phosphorylation, leaving them especially vulnerable to the mitochondrial damage caused, and it is for this reason that these tissues are the most severely affected by the pathogenesis of FRDA.

Cellular models of Friedreich's Ataxia have employed L-buthionine (S,R)-sulfoximine (BSO), a chemotherapeutic agent which blocks the rate limiting step of *de novo* glutathione (GSH) synthesis, catalyzed by gamma-glutamylcysteine synthetase. Studies have shown that donor fibroblasts from Friedreich's Ataxia patients are

extremely susceptible to this BSO-induced oxidative stress, while fibroblasts from healthy patients are not, due the presence of functional frataxin to absorb the increased load of cellular ROS when GSH is inhibited.

Currently, there are few effective treatment modalities for FRDA. Historically, treatment has been focused on palliative care: patient counseling, genetic counseling for prospective parents, speech therapy, physical therapy, wheelchair and other ambulatory device use, propranolol for tremors, dantrolene sodium for muscle spasms and symptomatic treatment for heart disease and diabetes. Recently, antioxidant and mitochondria specific iron chelation therapy have both been proposed as possible therapies to treat the root cause of FRDA. Iron chelation therapy works by a similar principal, removing the iron from around the mitochondria, preventing the formation of free radicals and preventing the associated mitochondrial damage.

The neuroprotective effects of 17β -estradiol (E2) have been clearly documented for more than a decade in a variety of disease states involving mitochondrial disruption, but the exact mechanism of action is currently poorly understood. Although the neuroprotective effects of estrogens have never been tested in an FRDA model and FRDA shows no gender-bias in incidence, some epidemiologic studies of FRDA have shown a better prognosis in female patients. Since there is a simple genetic test to determine the presence of FRDA in the children of silent FRDA carriers, it is possible to determine the presence of Friedreich's ataxia in newborns, years before the cardio- and neurodegeneration and clinical symptoms begin, a time window during which nonfeminizing estrogens and other antioxidants could potentially be clinically useful. Estrogens are putative candidate drugs to provide a neuroprotective effect in Friedreich's

ataxia. The ability of phenolic estrogens to protect against the oxidative damage of ROS, coupled with the possibility that they maintain the integrity of the oxidative phosphorylation process makes them ideal for the treatment of the underlying cellular dysfunction, not just the symptoms of FRDA.

This study will determine if E2 and estrogen-like compounds can protect human FRDA fibroblasts from oxidative insults *in vitro*. In addition, we will attempt to determine the exact mechanism by which E2 acts and investigate the possibility of any synergistic effects with other compounds proposed as putative treatments for FRDA.

ROLE OF ESTROGEN AND ESTROGEN ANALOGUES IN FRIEDREICH'S
ATAXIA CYTOPROTECTION

DISSERTATION

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS	5
LIST OF TABLES	8
LIST OF ILLUSTRATIONS	9
SPECIFIC AIMS	13
CHAPTER	
I. INTRODUCTION: FRIEDREICH'S ATAXIA & THE CASE FOR ANTIOXIDANTS	15
II. ESTROGEN PROTECTION IN FRIEDREICH'S ATAXIA SKIN FIBROBLASTS	30
III. R- AND S-EQUOL HAVE EQUIVALENT CYTOPROTECTIVE EFFECTS IN FRIEDREICH'S ATAXIA	58
IV. ESTROGEN PREVENTS OXIDATIVE DAMAGE TO THE MITOCHONDRIA IN FRIEDREICH'S ATAXIA SKIN FIBROBLASTS	74
V. SYNERGISTIC EFFECTS OF ESTROGEN AND METHYLENE BLUE IN FRIEDREICH'S ATAXIA	107
VI. CONCLUSIONS & FUTURE DIRECTIONS	129
APPENDIX	
I. FRIEDREICH'S ATAXIA FIBROBLASTS HAVE AN INCREASED VULNERABILITY TO BSO	138
II. ESTROGEN DOES NOT SIGNIFICANTLY INCREASE THE LEVEL OF FRATAXIN PROTEIN	147
III. ER β AGONISTS IN PHASE II CLINICAL TRIAL PROTECT	

FRIEDREICH'S ATAXIA FIBROBLASTS AGAINST BSO-INDUCED	
OXIDATIVE STRESS	156
REFERENCES	172

LIST OF TABLES

Chapter III

Table 1.	EC ₅₀ values for R- and S-equol for cell viability and ROS attenuation	72
----------	--	----

Chapter V

Table 1.	EC ₅₀ values for interaction studies	128
----------	---	-----

Chapter VI

Table 1.	Steroids used and compound properties	137
----------	---	-----

LIST OF ILLUSTRATIONS

Chapter I

Figure 1.	Proposed mechanism of E2 in BSO-treated FRDA fibroblasts	28
-----------	--	----

Chapter II

Figure 1.	Structures of E2, PPT, DPN, G1, ICI 182,780, G15, ZYC-26 and ZYC-23	49
Figure 2.	Cell viability in FRDA cells treated with BSO	50
Figure 3.	Cell viability in FRDA cells treated with BSO and E2	51
Figure 4.	Cell viability in FRDA cells treated with BSO and ZYC-26 or ZYC-23	52
Figure 5.	Cell viability in FRDA cells treated with BSO and PPT or DPN	53
Figure 6.	Cell viability in FRDA cells treated with BSO and G1 or E2 and G15	54
Figure 7.	DCFDA assay for ROS in FRDA cells treated with BSO and E2, PPT, DPN, ZYC-26 or ZYC-23	55
Figure 8.	Calcein AM staining in FRDA cells treated with BSO and E2, ZYC-26 ZYC-23, PPT, DPN, G1 or E2 and G15	56

Chapter III

Figure 1.	Structure of R- and S-Equol	69
Figure 2.	Cell viability in FRDA cells treated with BSO and R- or S-Equol	70
Figure 3.	DCFDA assay for ROS in FRDA cells treated with BSO and R- or S-Equol	71

Chapter IV

Figure 1.	Structures of E2, PPT, DPN, ZYC-26 and ZYC-23	97
Figure 2.	Western blot of ER α , ER β and GAPDH in FRDA cells	98
Figure 3.	Calcein AM staining in FRDA cells treated with BSO at 24, 36 and 48 hours and cell viability in FRDA cells treated with BSO and E2, PPT, DPN , ZYC-26 or ZYC-23	99
Figure 4.	8-isoprostane assay for lipid peroxidation in FRDA cells treated with BSO and E2, PPT, DPN , ZYC-26 or ZYC-23	100
Figure 5.	Aconitase activity assay in FRDA cells treated with BSO and E2, PPT, DPN , ZYC-26 or ZYC-23	101
Figure 6.	Seahorse XF24 assay for mitochondrial function and oxygen consumption rate in FRDA cells treated with BSO and E2, PPT, DPN , ZYC-26 or ZYC-23	102
Figure 7.	ATP concentration assay in FRDA cells treated with BSO and E2, PPT, DPN , ZYC-26 or ZYC-23	103
Figure 8.	FRET assay for mitochondrial membrane potential collapse in FRDA cells treated with BSO and E2, PPT, DPN , ZYC-26 or ZYC-23	104
Figure 9.	Proposed mechanism of E2 in BSO-treated FRDA fibroblasts	105

Chapter V

Figure 1.	Structures of E2, ZYC-26, MB and idebenone	123
Figure 2.	Cell viability in cells treated with BSO and E2 with MB	124
Figure 3.	Cell viability in cells treated with BSO and ZYC-26 with MB	125

Figure 4.	Cell viability in cells treated with BSO and E2 with idebenone	126
Figure 5.	Comparison of EC ₅₀ values for all compounds and interactions	
	Evaluated	127

Chapter VI

Figure 1.	Cell viability EC ₅₀ vs number of phenol rings for estrogens	136
-----------	---	-----

Appendix I

Figure 1.	Cell viability in FRDA and normal fibroblasts treated with BSO	145
Figure 2.	Calcein AM staining in FRDA and normal fibroblasts treated with BSO	146

Appendix II

Figure 1.	Western blot of frataxin in FRDA cells treated with 0, 1, 2, 3, 6, 12, 24 and 48 hours of estrogen	155
-----------	---	-----

Appendix III

Figure 1.	Structures of DPN, LY500307, raloxifene and tamoxifen	168
Figure 2.	Cell viability in FRDA cells treated with BSO and LY500307	169
Figure 3.	Cell viability in FRDA cells treated with BSO and DPN, LY500307, raloxifene, or tamoxifen	170
Figure 4.	Calcein AM staining in FRDA cells treated with BSO and DPN, LY500307, raloxifene, or tamoxifen	171

Specific Aims:

Specific Aim 1: To determine if phenolic estrogens protect human FRDA fibroblasts from BSO-induced oxidative stress. We will determine the effects of E2 as well as the ER α preferring agonist PPT and ER β preferring agonist DPN on cell viability in human FRDA fibroblasts exposed to BSO in the presence and absence of the nonselective ER antagonist ICI 182,780. This will allow us to determine whether estrogens are cytoprotective in this model, as well as the influence of individual estrogen receptors on this phenomenon. We will also examine the effects of ZYC-26 (a nonfeminizing, phenol ring containing estrogen) and ZYC-23 (a nonfeminizing estrogen with no phenol ring) to determine the influence of direct antioxidant properties of estrogen, imparted by the phenolic ring in their structure.

Specific Aim 2: To determine the mechanism for estrogen cytoprotection in human FRDA fibroblasts. We will test the hypothesis that the mechanism of estrogen protection is independent of any known ER, as well as confirm that estrogens act to prevent the accumulation of ROS. We will then test if estrogens are able to prevent lipid peroxidation and whether they can prevent the collapse of the mitochondrial membrane potential in the FRDA fibroblast model. We will also assess the levels of mitochondrial glutathione and iron levels. We will then assess the function of the mitochondria in this model by measuring intracellular ATP concentrations, overall oxygen consumption and extracellular acidification rates, and aconitase and individual ETC complex function, all in the presence of BSO and estrogen-like compounds.

Specific Aim 3: To determine if phenolic estrogen-like compounds act synergistically with methylene blue (MB). We will test the hypothesis that the antioxidant properties of E2 and estrogen-like compounds, including PPT, DPN, ZYC-26 and ZYC-23, will act synergistically with the antioxidant and oxidative phosphorylation-augmenting properties of MB. This will be measured in terms of potency and efficacy on cell viability of each estrogen compound alone and in combination with MB.

CHAPTER I

Friedreich's Ataxia: Disease Progression and Current Therapeutic Strategies

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Abstract

First described in 1863, Friedreich's Ataxia (FRDA) is a progressive hereditary neurodegenerative disorder inherited in an autosomal recessive manner. Caused by a trinucleotide repeat expansion effectively preventing the production of frataxin protein, this disease is characterized by progressive mitochondrial damage, resulting in cell death in organ systems most dependent on the mitochondria for energy production, principally the nervous system and heart. While the exact role of frataxin is currently still unknown, its absence results in the depression of electron transport chain respiration, impairment of function of iron-sulfur containing proteins and impairment of the intrinsic intracellular antioxidant systems. Herein, we review the cellular events that initiate widespread organ dysfunction and discuss ongoing research in therapeutics aimed at inhibiting this damage and halting or slowing the progression of FRDA, including those on estrogen treatment from our laboratory.

Outline:

1. Disease Mechanism & Clinical Symptoms
2. Treatment strategies
 - 2.1 Iron Chelators
 - 2.2 Antioxidants & Electron Transport Chain Modulators
 - 2.2 Frataxin Level Modifiers
3. Conclusions

Disease Mechanism & Clinical Symptoms

First described in a series of papers by Nikolaus Friedreich [Friedreich 1863a, 1863b, 1863c; 1876; 1877], Friedreich's ataxia (FRDA) affects 1 in 50,000 to 1 in 20,000 worldwide with a carrier rate of 1 in 120 to 1 in 60 making it the most common type of inherited ataxia worldwide [Bradley et al., 2000; Campuzano et al., 1996; Harding, 1983; Leone et al., 1990; Pandolfo, 1998; Schulz et al., 2009]. FRDA is caused by a progressive GAA trinucleotide repeat expansion in the first intron of the FXN gene on chromosome 9q13-21, responsible for producing the frataxin [Fujita et al., 1989; Hanauer et al., 1990], causing a self-associating complex of triple helical DNA to form and inducing histone deacetylation during DNA transcription [Grabczyk and Usdin, 2000; Heidenfelder, 2003; Sakamoto et al., 1999, 2001; Wells, 2008], preventing effective transcription and resulting in a loss of intracellular frataxin [Bradley et al., 2000; Campuzano et al., 1996; Harding, 1983; Lodi et al., 2006; Montermini et al., 1997]. The exact function of frataxin is currently unclear, although it has been suggested that it is required for iron-sulfur (Fe-S) cluster assembly [Adinolfi et al., 2009; Prisci et al., 2010; Tsai and Barondeau, 2010]. Its absence results in dysfunctional iron metabolism, with impaired function of iron-sulfur (Fe-S) cluster proteins, including heme, electron transport chain (ETC) proteins and the Krebs's cycle protein, aconitase, as well as dysregulation of the cellular redox state [Delatycki et al., 2000; Gakh et al., 2006; Lodi et al., 2006], ultimately leading to widespread progressive oxidative damage to many components of the cell, specifically the mitochondria [Karthikeyan et al., 2003]. The inhibition of Fe-S containing protein function severely impairs cellular respiration [Bradley et al., 2000; Bulteau et al., 2004; Rötig et al., 1997], which is further complicated by simultaneous

oxidative damage to these same mitochondrial proteins [Al-Mahdawi et al., 1997; Bulteau et al., 2004; Chantrel-Groussard et al., 2001; Gakh et al., 2006]. This mitochondrial damage prevents the cell from being able to produce enough ATP to match its energetic needs, resulting cell death [Jauslin et al., 2002, 2003; Santos et al., 2010], a disease mechanism common to many neurological and neurodegenerative diseases, including ischemic stroke, Alzheimer's disease and Parkinson's disease [Beal, 2000; Gibson et al., 1998; Lenaz et al., 2006, 2010; Mizuno et al., 1989; Parker et al., 1989; Simpkins and Dykens, 2008].

The length of the GAA triplet repeats, specifically the shorter of the two GAA repeat alleles, is inversely proportional to the level of frataxin protein present in the cell and the age of disease onset, and is directly proportional to the severity of clinical symptoms [Campuzano et al., 1997; Dürr et al., 1997; Isnard et al., 1997]. Studies in yeast have shown that the depletion of frataxin homologues is related to oxidative damage and results in progressive accumulation of mitochondrial damage [Karthikeyan et al., 2003]. Cells and tissues most dependent on aerobic respiration and oxidative phosphorylation for ATP production are the first to succumb to the oxidative damage, including neurons in the brain and spinal cord, cardiomyocytes and pancreatic beta cells. It is still unclear why there is variable cell death and dysfunction within these tissues, including why certain spinal cord tracts are affected and others are not. It should also be noted that there is a phenotypic contribution to the disease process of cells in these organ systems that are dysfunctional but still viable [Santos et al., 2010].

Although FRDA can be diagnosed with genetic tests before birth [Monros et al., 1995; Pandolfo and Montermini, 1998; Wallis et al., 1989], FRDA usually presents

clinically around puberty, although the onset can vary from infancy to the third decade of life [Dürr et al., 1996; Lodi et al., 2006]. The presenting symptoms are usually related to gait and other motor symptoms, leading to progressive ataxia [Dürr et al., 1996; Harding, 1981; Harding, 1983]. There is a degeneration of the dorsal root ganglia (DRG), followed by cranial nerve and ascending spinal cord tracts, including the spinocerebellar and corticospinal tracts and posterior columns, resulting in hearing and ocular abnormalities, tremor, ataxia, weakness and sensory abnormalities [Al-Mahdawi et al., 2006; Dürr et al., 1996; Geoffroy et al., 1976; Lodi et al., 2006; Harding et al., 1983]. Later, extra-neurological symptoms are common, including pes cavitus, lateral and kyphoscoliosis and an increased incidence of type 1 diabetes [Al-Mahdawi et al., 2006; Dürr et al., 1996; Geoffroy et al., 1976; Harding, 1981; Harding, 1983]. There is also a 66-91% incidence of cardiac symptoms, including hypertrophic cardiomyopathy with interstitial fibrosis, the most common cause of premature death in FRDA patients [Al-Mahdawi et al., 2006; Dürr et al., 1996; Dutka et al., 2000; Geoffroy et al., 1976; Harding, 1983; Isnard et al., 1997].

Treatment Strategies

Iron Chelators

Since there is a wealth of evidence detailing dysregulation of iron in FRDA patients and cellular models, drugs that have the potential for iron chelation have been investigated for use in FRDA [Lodi et al., 2006; Santos et al., 2010]. The accumulation of intracellular iron, specifically around the mitochondria, increases the levels of ROS and inhibits electron transport chain complexes in the heart [Rustin et al., 1999], further inhibiting mitochondrial energy production [Bradley et al., 2000; Bulteau et al., 2004; Rötig et al., 1997]. One such drug, deferoxamine, is able to chelate iron in cell culture, attenuating the reduction of activity of mitochondrial complexes; however aconitase and frataxin mRNA and protein levels are reduced [Li et al., 2008], making it a poor choice for FRDA treatment. Iron chelators that specifically target the mitochondria [Richardson, 2003; Lodi et al., 2006] such as deferiprone are currently being evaluated *in vitro* [Goncalves et al., 2008; Kakhlon et al., 2008] and in clinical trials [Boddaert et al., 2007]. Trials with this compound have yielded conflicting results, with some studies showing that deferiprone reduces ROS-induced damage and protects the mitochondria [Kakhlon et al., 2008], while others have suggested that it inhibited aconitase activity [Goncalves et al., 2008]. Clinical trials have shown that deferiprone reduces iron buildup in certain brain regions with possibly improved neurological function [Boddaert et al., 2007]. Compounds with phenol rings in their structure have long been known to act as iron chelators [Moeller and Shellman, 1953], and this property may prove to be partially responsible for their efficacy in protecting cell viability in FRDA fibroblast cultures [Richardson et al., 2011, 2012].

Antioxidants & Electron Transport Chain Modulators

For over a decade, much of the work in FRDA treatment that has been done has involved the prevention of oxidative mitochondrial damage and the maintenance of mitochondrial oxidative phosphorylation function [Santos et al., 2010]. Studies have shown that in addition to the intracellular mechanism of FRDA, patients with the disease demonstrate impaired manganese superoxide dismutase [Pandolfo, 2002], and have generally increased levels of lipid peroxidation [Emond et al., 2000], general oxidative stress and DNA damage [Schulz et al., 2000]. Idebenone, currently the first drug to reach Phase III clinical trials for FRDA, is a CoQ₁₀ equivalent that acts by both decreasing intracellular reactive oxygen species and by acting as an electron shuttle between the damaged complex I and III in the ETC, promoting oxidative phosphorylation in the face of impaired mitochondrial complexes [Meier and Buyse, 2009; Rustin et al., 1999]. This strategy allows the cells to continue aerobically producing high levels of ATP even with reduced ETC complex activity. Clinical studies have found that idebenone is able to decrease lipid peroxidation and other markers of oxidative stress in the heart and decrease the extent of hypertrophic cardiomyopathy [Rustin et al., 1999; Schulz et al., 2000] and one study showed a small improvement in ataxia [Di Prospero et al., 2007], although there was only a very modest increase in lifespan associated with this treatment. It is possible that the limited success on nervous function was due to the initiation of treatment too late in the disease course to prevent neuron death or lower CNS penetration relative to cardiac levels. In early 2011, idebenone failed its phase III clinical trial on the basis that it did not sufficiently improve cardiac outcomes in FRDA patients as measured by cardiac output or left ventricular hypertrophy [Lagedrost et al., 2011].

A recent study has focused on methylene blue (MB) as a potential therapy for FRDA. Methylene blue, a drug used variously over the past century for cyanide and carbon monoxide poisoning, ifosfamide neurotoxicity, methemoglobinemia and malaria, has much the same neuroprotective mechanism of action as idebenone: it acts as both an antioxidant and promotes oxidative phosphorylation, serving as an electron carrier shuttling electrons from NADH to cytochrome c in the presence of complex I/III damage [Wen et al., 2011]. Studies in fibroblasts have shown that MB is effective in attenuating ROS, increasing intracellular ATP concentrations, enhancing oxidative phosphorylation function, and preventing cell death in an FRDA fibroblast cell model [Yu et al., 2011]. Other antioxidants have also been effective in the FRDA fibroblast model. Jauslin et al., have demonstrated that antioxidants, specifically those targeted to the mitochondria are able to prevent cell death in FRDA fibroblasts [Jauslin et al., 2002, 2003], and that a novel compound with the active groups of idebenone and vitamin E, Fe-Aox29, is more potent than either idebenone or vitamin E alone or in combination [Jauslin et al., 2007].

Our lab has focused on the antioxidant potential of phenolic estrogens in treating the ROS-mediated component of FRDA. We have shown that in FRDA fibroblasts, independent of any known estrogen receptor, estrogen and estrogen-like compounds, including several non-feminizing estrogens, are able to attenuate ROS and prevent cell death [Richardson et al., 2011] through a phenol-quinol cycling antioxidant mechanism [Prokai et al., 2003; Prokai-Tatrai et al., 2008]. The potency and efficacy of these compounds depended on having at least one phenol ring in the molecular structure, with potency being correlated to the number of phenol rings [Behl et al., 1997; Moosmann and Behl, 1999; Richardson et al., 2011]. In addition, the EC₅₀ values for this set of

compounds were in the low nanomolar range [Richardson et al., 2011], significantly lower than idebenone, vitamin E or tailor-made antioxidants such as Fe-Aox29 [Jauslin et al., 2007]. Further studies have shown that these phenol ring containing estrogens are able to prevent lipid peroxidation, maintain aconitase activity, mitochondrial function and ATP production in FRDA fibroblasts (Fig. 1) [Richardson et al., 2012].

Newly developed non-feminizing estrogens are particularly good candidates for neuroprotection as they are ideally suited to penetrate the blood brain barrier and insert into lipid membranes preventing the sequence of oxidative to intracellular molecules and organelles [Behl, 2002; Behl and Manthey, 2000; Behl and Moosmann, 2002; Behl et al., 1995; Rupprecht and Holsboer, 1999; Simpkins et al., 2008, 2010; Walf et al., 2011], without estrogen receptor binding ability or feminizing effects in vivo [Simpkins et al., 2010; Yi et al., 2011]. Since FRDA can be detected genetically before birth in the offspring of known carriers [Monros et al., 1995; Pandolfo and Montermini, 1998; Wallis et al., 1989], there is a window of opportunity for treatment before symptoms begin. During this time frame, beginning therapy with antioxidant molecules could potentially be very beneficial for preventing or delaying the disease process, rather than attempting to improve or suppress symptoms and recover from the neuronal damage and death later in life.

Frataxin Level Modifiers

Another strategy in treating FRDA has been to correct the frataxin deficiency underlying the molecular disorder of FRDA. The disorder is caused by the lack of frataxin, caused by the GAA trinucleotide repeat section in the first intron of the gene on chromosome 9q13-21 [Fujita et al., 1989; Hanauer et al., 1990], resulting in a self-associating complex of triple helical DNA to form preventing effective DNA transcription [Bradley et al., 2000; Campuzano et al., 1996; Grabczyk and Usdin, 2000; Harding, 1983; Lodi et al., 2006; Montermini et al., 1997; Sakamoto et al., 1999, 2001; Wells, 2008]. This strategy is particularly appealing since murine models indicate that a compound may only have to raise intracellular frataxin concentration to ~25% of normal levels to prevent cellular abnormalities and neuropathological findings [Miranda et al., 2002]. Several compounds have been tested to increase frataxin levels. Histone deacetylase inhibitors such as BML-210 have been found to increase frataxin levels in FRDA carriers and mice [Herman et al., 2006; Rai et al., 2008]. Recombinant erythropoietin (EPO) has also been found to significantly increase frataxin protein levels in FRDA fibroblasts [Sturm et al., 2005], although no increase in mRNA levels was observed indicating that this effect is due primarily to post-transcriptional effects [Acquaviva et al., 2008]. In our lab, estradiol was not observed to increase the levels of frataxin protein in the FRDA fibroblast cell model [Richardson and Simpkins, unpublished observations], although the estrogen effect in these cells is ER-independent. In neural or cardiac tissue containing ER α or ER β , there is a possibility that in addition to the observed antioxidant effects of estrogen [Richardson et al., 2011], there may also be

an ER-dependent effect, including a transcriptional effect involving the production of extra frataxin or other protective proteins.

Conclusions

FRDA is a mitochondrial disease that affects tissues that are most dependent on aerobic respiration, principally the CNS and heart [Al-Mahdawi et al., 2006; Dürr et al., 1996; Dutka et al., 2000]. The root cause of the disease is the absence of functional frataxin, resulting in iron dysregulation and oxidative damage to lipids and proteins, ultimately disrupting mitochondrial function [Karthikeyan et al., 2003; Pandolfo, 1998, 2002], similar to other neurodegenerative disorders such as Alzheimer's and Parkinson's diseases [Beal, 2000; Gibson et al., 1998; Lenaz et al., 2006, 2010; Mizuno et al., 1989; Parker et al., 1989; Simpkins and Dykens, 2008]. Antioxidant and mitochondrial treatment of FRDA has been met with modest success, with idebenone decreasing cardiac complications and slightly prolonging life [Di Prospero et al., 2007; Rustin et al., 1999; Schulz et al., 2000]. Since FRDA can be diagnosed before birth [Monros et al., 1995; Pandolfo and Montermini, 1998; Wallis et al., 1989], drugs designed to penetrate the blood brain barrier, including those based on steroid structures, and increase frataxin levels, attenuate ROS or support oxidative phosphorylation could be applied in this brief symptom-free time window to prevent or delay the devastating effects of FRDA and neuronal death, rather than try to control symptoms or reverse the disease process at a later date.

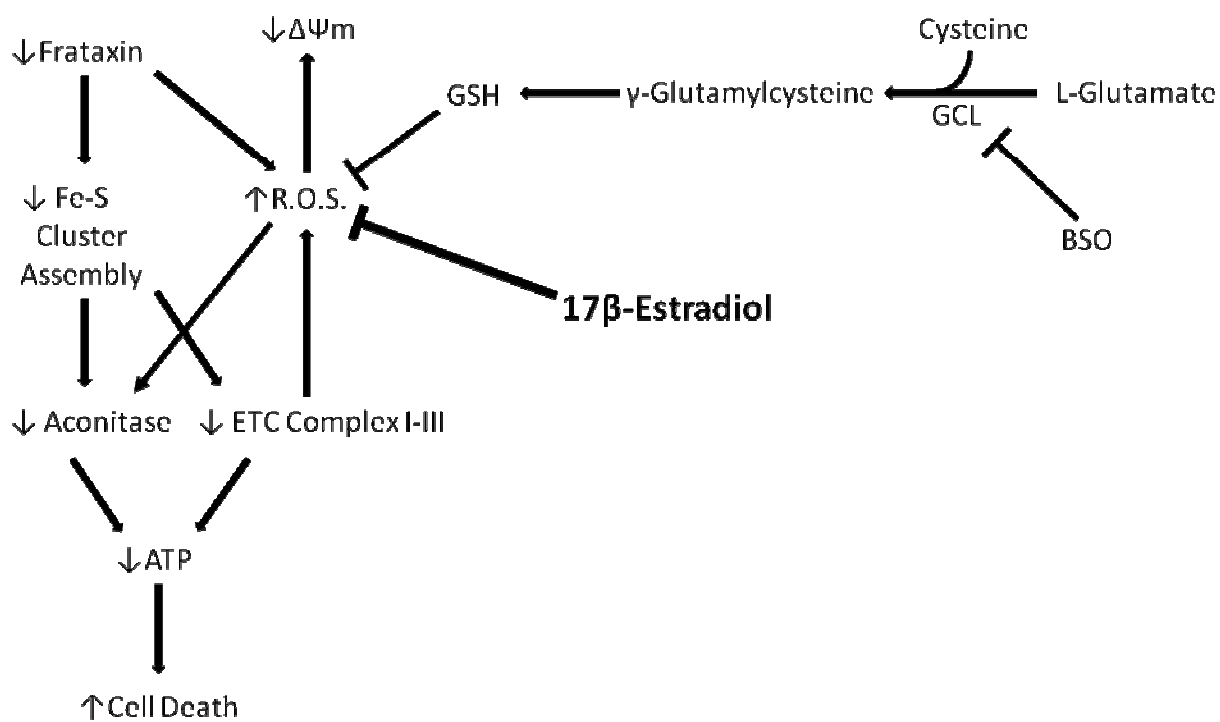


Figure 1. Proposed mechanism of 17 β -Estradiol in BSO-treated FRDA fibroblasts.

PREFACE TO CHAPTER II

Herein we examine the potency and efficacy of 17 β -Estradiol and several estrogen analogues, PPT (a triphenolic ER α agonist), DPN (a biphenolic ER β agonist), G1 (a nonphenolic mER agonist), ZYC-26 (a phenolic compound with no affinity for any known ER) and ZYC-23 (a nonphenolic compound with no affinity for any known ER), on cell viability in an *in vitro* cell model of Friedreich's ataxia. In addition we examine many of these estrogen-like compounds in the presence and absence of ER antagonists to determine the portion of the compound structure important to cytoprotection in this cell model.

CHAPTER II

Estrogen Protection in Friedreich's Ataxia Skin Fibroblasts

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Key Words: estrogens, 17 β -estradiol, antioxidants, Friedreich's Ataxia, fibroblasts, estrogen receptors

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Abstract

Estrogens have been shown to have protective effects on a wide range of cell types and animal models for many neurodegenerative diseases. The present study demonstrates the cytoprotective effects of 17 β -estradiol (E2) and estrogen-like compounds in an *in vitro* model of Friedreich's Ataxia (FRDA) using human donor FRDA skin fibroblasts. FRDA fibroblasts are extremely sensitive to free radical damage and oxidative stress, produced here using L-buthionine (S,R)-sulfoximine (BSO) to inhibit *de novo* glutathione (GSH) synthesis. We have shown that the protective effect of E2 in the face of BSO-induced oxidative stress is independent of ER α , ER β or GPR30 as shown by the inability of either ICI 182,780 or G15 to inhibit the E2-mediated protection. These cytoprotective effects appear to be dependent on antioxidant properties and the phenolic structure of estradiol as demonstrated by the observation that all phenolic compounds tested were protective, while all non-phenolic compounds were inactive, and the observation that the phenolic compounds reduced the levels of reactive oxygen species (ROS), while the non-phenolic compounds did not. These data show for the first time that phenolic E2-like compounds are potent protectors against oxidative stress-induced cell death in FRDA fibroblasts and are possible candidate drugs for the treatment and prevention of FRDA symptoms.

Introduction

Friedreich's Ataxia (FRDA) is the most common form of inherited ataxia in the world, affecting 1:20,000-1:50,000 people in the United States [Bradley et al., 2000; Harding, 1983; Leone et al., 1990; Pandolfo, 1998; Schulz et al., 2009]. It is inherited in an autosomal recessive manner due to a GAA trinucleotide repeat expansion in the first intron of the FXN gene on chromosome 9q13-21, causing gene silencing and a functional absence of the mitochondrial-localizing protein frataxin [Bradley et al., 2000; Campuzano et al., 1996; Harding, 1983; Isnard et al., 1997; Lodi et al., 2006; Montermini et al., 1997]. The frataxin protein is responsible for removing iron from around the mitochondria, preventing the formation of reactive oxygen species (ROS) [Al-Mahdawi et al., 2006; Babcock et al., 1997; Bradley et al., 2000; Jauslin et al., 2003]. The loss of FXN function causes an accumulation of mitochondrial iron, reactive oxygen species (ROS) and impaired function of Fe-S centers in mitochondrial proteins, leading to mitochondrial damage and a decrease in activity of mitochondrial complexes I-III [Bradley et al., 2000; Lodi et al., 2006; Jauslin et al., 2002, 2003; Rötig et al., 1997]. The damaged mitochondria is unable to match ATP production to the cell's energy requirements, a common mechanism of cell death in a wide range of neurologic disorders including Alzheimer's disease, Parkinson's disease and Huntington's disease [Gibson et al., 1998; Lenaz et al., 2006, 2010; Mizuno et al., 1989; Simpkins and Dykens, 2008]. FRDA classically presents with a degeneration of the spinocerebellar tracts and posterior columns of the spinal cord, resulting in gait ataxia and tremor [Al-Mahdawi et al., 2006; Dürr et al., 1996; Geoffroy et al., 1976; Lodi et al., 2006]. Also present are pes cavitus, speech problems, lateral and kyphoscoliosis, tremor, weakness, diabetes and a 91% incidence of heart disorders, such as hypertrophic cardiomyopathy with interstitial

fibrosis, which is the most common cause of early death in FRDA patients [Al-Mahdawi et al., 2006; Dürr et al., 1996; Dutka et al., 2000; Geoffroy et al., 1976; Harding, 1981, 1983].

The neuroprotective effects of 17 β -estradiol (E2) have been clearly documented for more than a decade [Behl et al., 1995; Bishop and Simpkins, 1994; Goodman et al., 1996; Singer et al., 1996] in a variety of disease states involving mitochondrial disruption, but the exact mechanism of action is currently poorly understood [Simpkins and Dykens, 2008; Simpkins et al., 2008, 2009]. Estrogen has been demonstrated to have antioxidant properties [Perez et al., 2006; Prokai et al., 2003; Prokai-Tatrai, et al., 2008; Wang et al., 2001; Wang et al., 2003], modulate Ca²⁺ flux [Sarkar et al., 2008], stabilize mitochondrial membrane potential ($\Delta\Psi_m$) [Simpkins and Dykens, 2008; Wang et al., 2001; Wang et al., 2003], maintain the activity of electron transport chain (ETC) complexes I, III and IV, helping to maintain aerobic ATP production [Wang et al., 2001; Jayachandran et al., 2010], and promote a favorable balance of anti-apoptotic:pro-apoptotic proteins in the cell [Simpkins and Dykens, 2008; Simpkins et al., 2009]. Although the neuroprotective effects of estrogens have never been tested in an FRDA model and FRDA shows no gender-bias in incidence [Dürr et al., 1996; Geoffroy et al., 1976], epidemiologic studies of FRDA have shown a better prognosis in female patients [Pandolfo, 1998] and the proposed mitochondrial-support mechanisms of E2 suggests a possible role for E2 in the prevention and treatment of FRDA symptoms [Dykens et al., 2005].

In the present study, we show that estrogens increase cell viability in FRDA fibroblasts subjected to oxidative stressors in a manner that is independent of any known

estrogen receptors (ER). In the FRDA fibroblast model, the ability of E2 to provide protection from the pro-oxidant stressors is dependent on the presence of a phenol ring, and the potency of each compound is in part related to the number of phenol groups present in the compounds. This property makes estrogens good candidates to join the list of cellular antioxidants proposed to alleviate Friedreich's ataxia symptoms and modify the disease process [Jauslin et al., 2003].

Methods

Cell Culture. Fibroblasts from a 30 year old Friedreich's Ataxia (FRDA) patient (Coriell Institute, Camden NJ, USA) were maintained in Dulbecco's Modified Eagle Medium (DMEM; ThermoScientific, Waltham, MA, USA) with 10% charcoal-stripped fetal bovine serum (CSFBS; ThermoScientific, Waltham, MA, USA), 1% GlutaMAX (ThermoScientific, Waltham, MA, USA) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C in 90% humidity and 5% CO₂. At the time of treatment, the FRDA fibroblast media was changed to phenol red- and sodium pyruvate-free DMEM (ThermoScientific, Waltham, MA, USA) and 1% penicillin-streptomycin. All experiments were conducted using cell passages 15-22.

Chemicals & Reagents. 17 β -Estradiol (E2) was acquired from Steraloids, Inc. (Newport, RI, USA). L-buthionine (S,R)-sulfoximine (BSO) was obtained from Sigma-Aldrich (St Louis, MO, USA). G1, an agonist at the membrane estrogen receptor GPR30, and G15, an antagonist at GPR30, were obtained from CalBiochem (San Diego, CA, USA). ICI 162,780, 4,4',4'-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT) and diarylpropionitrile (DPN) were purchased from Tocris Bioscience (Ellisville, MO, USA). ZYC-26 and ZYC-23 were synthesized in the laboratories of Dr. Douglas Covey [Perez et al., 2006]. Structures for all steroids are provided in Figure 1 and were drawn using ChemDraw software.

Treatment Paradigm. FRDA fibroblasts were removed from culture with 0.25% Trypsin-EDTA (Invitrogen, Carlsbad, CA, USA) and plated on 96-well plates at a density

of 3000 cells per well in DMEM with 10% CSFBS, 1% GlutaMAX and 1% penicillin-streptomycin. After 24 hours the media was removed and replaced with phenol red- and sodium pyruvate-free DMEM with 1% penicillin-streptomycin. The cells were then treated for 48 hours with either dimethyl sulfoxide vehicle control (DMSO; Sigma-Aldrich, St Louis, MO, USA) or 1mM BSO in the presence of 1nM-10 μ M E2, Diarylpropionitrile (DPN), 4,4',4'-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT), 2-adamantyl, 4 methyl estrone (ZYC-26), 2-adamantyl, 3-O-methyl estradiol (ZYC-23) or G1, concentrations of estrogens known to be neuroprotective in various cell lines [Yi et al., 2008], with and without the ER α and ER β receptor antagonist ICI 182,780 and the membrane ER receptor (GPR30) antagonist G15.

Lactate Dehydrogenase (LDH) Cell Viability Assay. After 48 hours of treatment 50 μ L of media was removed from each of the wells of the 96-well treatment plate and placed in a separate 96-well plate. 100 μ L of a solution consisting of 12mL of 200 mM pH 8.2 Tris(hydroxymethyl)aminomethane hydrochloride (Sigma-Aldrich, St Louis, MO, USA) with 50 μ L lactic acid and 4.2 mg iodonitrotetrazolium chloride (INT; Sigma-Aldrich, St Louis, MO, USA), 1.1 mg phenazine methosulphate (PMS; Sigma-Aldrich, St Louis, MO, USA) and 10.8 mg β -nicotinamide adenine dinucleotide hydrate (NAD; Sigma-Aldrich, St Louis, MO, USA) is added to each well. The absorbance of the resulting reaction was read with a Tecan Infinite M200 plate reader at 490nm and recorded once the reaction is linear for greater than 2 minutes. Cell viability for each well (WellX) was determined by: $100 - (100 * (\text{WellX} - \text{media}) / (0.1\% \text{ TritonX100} - \text{media}))$. Measurements were then confirmed by visual inspection of the FRDA fibroblasts.

Reactive Oxygen Species Assay. After 12 hours of treatment the media was removed from each well of the 96-well plate, and 100 μ L of a 1 μ M 2',7'-Dichlorodihydrofluorescein diacetate (DCFDA; AnaSpec Inc., Fremont, CA, USA) in phosphate buffer (PBS) was added to each well. The plates were returned to a 37°C incubator for 20 minutes, then each well was washed three times with PBS and the resulting reaction was read on a Tecan Infinite M200 plate reader with an absorbance of 495 nm and an emission of 529 nm.

Calcein AM Cell Imaging. Cells were plated on a 96-well plate at a density of 5,000 cells per well, then treated identically to those in the LDH cell viability assay. After 48 hours of BSO and steroid treatment, the media was removed from each of the wells of a 96-well treatment plate. 100 μ L of a 1 μ g/mL Calcein AM (CalBiochem, San Diego, CA, USA) in phosphate buffer pH 7.2 (PBS; Fisher Scientific, Pittsburg, PA, USA) was added to each of the wells and the plate was incubated for 10 minutes at 37 °C. The cells were then photographed using a Zeiss Axio Observer Z1 inverted microscope and Zeiss AxioVision 4.6 image processing software.

Data and Statistics. All data are displayed as mean \pm 1 standard deviation. These data were analyzed using the ANOVA on the SYSTAT[®] program with Tukey's post hoc test for statistical evaluation against an alpha level of 0.05. All bar graphs were made using GraphPad Prism 5 and EC₅₀ calculations were made with GraphPad Prism 5. For all groups, n=8 wells and experiments were repeated three times to ensure consistency.

Results

Effects of BSO on cell viability FRDA fibroblasts. We determined the relative vulnerability of human donor FRDA fibroblasts to the effects of GSH depletion. We used 100 μ M to 10 mM BSO for 48 hours to inhibit the rate limiting enzyme in the *de novo* synthesis of GSH, γ -glutamyl cysteine synthase. There was a significant reduction in cell viability of FRDA fibroblasts in the presence of BSO. Viability was reduced from $96.3 \pm 1.2\%$ in the vehicle control to $74.7 \pm 2.8\%$, $44.7 \pm 5.0\%$ and $32.3 \pm 4.1\%$ in the presence of 100 μ M, 1 mM and 10 mM BSO, respectively (Fig. 2). Inasmuch as 1 mM BSO reduced cell viability by about 60%, we used this concentration to assess the cytoprotective activity of each of the compounds.

Effects of 17 β -estradiol on cell viability in BSO-treated FRDA fibroblasts. To determine if E2 exhibited a protective effect against the oxidative damage allowed by BSO-induced glutathione depletion, we tested 10 nM-10 μ M E2 in cells treated with 1mM BSO (Fig. 3). E2 produced a significant increase in cell viability ($p < 0.05$) in concentrations ranging from 100 nM to 10 μ M ($n=8$), with an EC_{50} of 15.5 nM, and near maximum effects at 1 μ M E2. To determine if these cytoprotective effects were due to either ER α or ER β , we used 500 nM ICI 182,780, a concentration 1500-fold greater than the 0.29 nM IC_{50} of ICI 182,780 at both ER α and ER β [de Cupis et al., 1995], in conjunction with 1 mM BSO and 100 nM E2. ICI 182,780 did not significantly reduce the viability of the FRDA fibroblasts as compared to 1 mM BSO and 100 nM E2 treatment. In addition, the lack of effect of effect ICI 182,780 was observed when E2 and

ICI 182,780 were given as a 24-hour pretreatment (unpublished observations). These data indicate that E2 acts by a mechanism which is independent of either ER α or ER β .

Effects of ICI 182,780 on cell viability in BSO-treated FRDA fibroblasts. At 500 nM, ICI 182,780 alone provided no significant increase in cell viability against 1mM BSO treatment. However at 2 μ M, a concentration about 7000 times the IC₅₀ of ICI 182,780 at ERs, there was a statistically significant increase in cell viability (data not shown). These data are consistent with observations that the protective effects of E2 are due to the phenol ring, included in the structure of both E2 and ICI 182,780 (Fig. 1), and unrelated to either ER α or ER β .

Effects of non-feminizing estrogens on cell viability in BSO-treated FRDA fibroblasts. ZYC-26, a non-feminizing estrogen, has cytoprotective properties in this FRDA fibroblast cell model with statistically significant effects at concentrations ranging from 10 nM to 10 μ M (Fig. 4A). While unable to bind to either ER α or ER β , ZYC-26 has a phenolic ring as part of its structure, giving it antioxidant properties similar to E2 (Perez et al., 2006) (Fig. 1). At 100 nM, ZYC-26 increases the cell viability from $33.8 \pm 7.59\%$ to $80.37 \pm 3.8\%$, with an EC₅₀ of 23.1 nM. Conversely, ZYC-23, which does not bind to estrogen receptors [Perez et al., 2006], had no significant effects on cell viability across the 10 nM-10 μ M concentration range tested (Fig. 4B). These data support the hypothesis that the phenol ring is essential in the protective effects of estrogens and estrogen-like compounds as ZYC-23 has an O-methyl at the 3-position of the phenol ring, eliminating the antioxidant properties of the compound [Perez et al., 2006] (Figs. 1 and 8). As is

evident in Figure 4, the phenol ring is essential for maintenance of cell viability in the fibroblast FRDA disease model.

The effects of ER α and ER β preferring agonists on cell viability in BSO-treated

FRDA fibroblasts. PPT is a relatively ER α specific agonist that has cytoprotective effects in this cell model in the concentration range of 10 nM to 10 μ M (Fig. 5A). This compound appears to have greater protective potency than E2. DPN is a relatively selective ER β agonist with protective effects against BSO-induced cytotoxicity at concentrations ranging from 100 nM to 10 μ M (Fig. 5B). Although these compounds are specific for individual ERs, the effect of neither compound was inhibited by ICI 182,780, indicating that these compounds act through a mechanism other than their respective estrogen receptors. Structures of the 2 compounds (Fig. 1) shows that the ER α agonist PPT has 3 phenol rings and the ER β agonist DPN has 2 phenol rings, suggesting a possible ER-independent mechanism by which these 2 compounds provide cytoprotection to FRDA fibroblasts in the presence of 1 mM BSO. The extra phenol rings in the PPT molecule also provide a potential explanation for the observation that PPT has significant protective effects at 10 nM with an EC₅₀ of 4.6 nM, while E2 doesn't protect against the BSO insult until it reaches 100 nM concentrations, and has an EC₅₀ of 15.5 nM. The extra phenol rings on the DPN molecule also seem to provide additional protective potency, giving it an EC₅₀ value that falls between E2 and PPT at 9.3 nM.

Membrane estrogen receptor (mER/GPR30) effects on cell viability. GPR30 is a proposed membrane-associated G_q-protein coupled estrogen receptor. To assess estrogen

actions through this receptor we tested a range of concentrations of G1, a GPR30 preferring ER agonist. As seen in Figure 6A, there were no significant protective effects at any tested concentration of G1. In addition, the GPR30 antagonist G15, at concentrations ranging from 1 nM to 10 μ M, did not antagonize the cytoprotective effects of 100nM 17 β -estradiol (Fig. 6B). These data indicate that the membrane associated estrogen receptor GPR30 is not involved in the increase in cell viability seen with E2 in FRDA fibroblasts exposed to 1 mM BSO (Fig. 3).

The effects of estrogen-like compounds on BSO-induced reactive oxygen species (ROS) formation. We first determined the time-course of ROS formation following BSO administration to FRDA fibroblast. At 1, 2, 3, 6, 12, 18 and 24 hours after BSO treatment of FRDA fibroblasts, ROS was determined using a DCFDA assay. Peak levels of ROS were seen at 12 hours of treatment (data not shown). We therefore assessed the effects of compounds on ROS at 12 hours after BSO and estrogen treatment.

To determine if the phenol groups present in these estrogen compounds were indeed exerting an antioxidant activity, we measured ROS following BOS in the presence or absence of E2, PPT, DPN, ZYC-26 and ZYC-23 at 100 nM, a concentration that produced near-maximal effects in the phenolic compounds. As can be seen in Figure 7, BSO doubled ROS concentrations. Those compounds with phenol rings were effective in preventing this BSO-induced rise in ROS, whereas ZYC-23 provided no significant effect. In addition, the group treated with 1 mM BSO and 100 nM PPT, an estrogen-like compound with 3 phenol rings (Fig. 1), had significantly lower ROS concentrations than

either the group treated with 1mM BSO and 100 nM E2 or the DMSO control group (Fig. 7), observation consistent with the enhanced potency of PPT.

Effects of estrogens on BSO-induced cell death as measured by the Calcein AM Assay. To achieve a visual confirmation of the damaging effects of BSO and the protective effects of phenolic estrogens, FRDA fibroblasts were treated with BSO (1 mM) with or without simultaneous treatment with each estrogen (100 nM) for 48 hours. Cells were then stained with Calcein AM, which stains live cells (31) and photomicrographs were taken. As shown in Figure 8, the results obtained with the Calcein AM assay were consistent with those obtained with the LDH viability assay.

Discussion

The potential role of estrogens in protection against various neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, Huntington's disease and ischemic stroke has been known for over a decade [Behl et al., 1995; Bishop and Simpkins, 1994; Goodman et al., 1996; Simpkins et al., 1997; Singer et al., 1996]. The proposed mechanisms by which estrogens provide neuroprotection are varied [for review see Simpkins and Dykens, 2008], but a clear case has been made for their therapeutic efficacy in neurodegenerative disorders. Previously, it was unknown if estrogens and estrogen-like compounds had any effect on the pathogenesis of Friedreich's ataxia, although the observation that females tend to have less severe symptoms and a later onset of the disease process [Pandolfo, 1998] suggests that there is a beneficial effect of estrogens in this disease process. For the first time, this study presents data showing that estrogens and estrogen-like compounds containing a phenol ring are protective against oxidative stress in FRDA cells, and that this process is not mediated by any known estrogen receptor.

Friedreich's ataxia (FRDA) is the most common form of inherited ataxia in the world, caused by an autosomal recessive trinucleotide repeat expansion in the first intron of the FXN gene on chromosome 9q13-21 [Bradley et al., 2000; Harding, 1983; Isnard et al., 1997; Leone et al., 1990]. FRDA fibroblasts were used in this study were taken from a skin-punch biopsy of a 30 year old male Caucasian patient with clinical Friedreich's ataxia symptoms. The cells are homozygous for the GAA expansion in the first intron of the frataxin gene with 541 trinucleotide repeats on one allele and 420 on the second. Human fibroblasts contain both estrogen receptor alpha and beta [Haczynski et al., 2002]

as well as the membrane associated estrogen receptor GPR30 [Madeo and Maggiolini, 2010]. Human FRDA fibroblasts have been used as a cell model in Friedreich's ataxia studies by several groups for the past 10 years, and have become a standard cell type to use in studying this disease process [Jauslin et al., 2002, 2003, 2007; Li et al., 2008; Ku et al., 2010]. FRDA fibroblasts are extremely vulnerable to oxidative damage caused by glutathione depletion with L-buthionine (S,R)-sulfoximine treatment as compared to genetically normal fibroblasts, which require a much greater dose of BSO to obtain a comparable toxicity [Jauslin et al., 2002], and it has been shown that antioxidants, specifically those targeted at the mitochondria are effective at protecting the FRDA fibroblasts from oxidative stress [Jauslin et al., 2003]. FRDA fibroblasts, while not primary neurons or cardiocytes, are an accepted model for Friedreich's Ataxia as a disease process [Jauslin et al., 2002, 2003]. It is thought that the oxidative damage induced by the depletion of glutathione by BSO in this cell type may be analogous to the pathogenic process that is occurring in both the CNS and heart, leading to the clinical symptoms of Friedreich's ataxia [Bradley et al., 2000; Lodi et al., 2006].

In this study, we have shown that estrogen-related compounds are able to protect FRDA fibroblast cell from BSO-induced oxidative damage. These effects are independent of the action on ER α , ER β or GPR30 as indicated by the lack of inhibition of the cytoprotection induced by E2, ZYC-26, PPT or DPN by ICI 182,780 or G15 and the lack of efficacy of G1 in promoting cell viability. It appears that the potential protective effects of estrogen-like compounds are dependent on its intrinsic antioxidant properties as determined by the inclusion of a phenol ring in the molecule's structure (Figs. 1 and 7). The protective effect of antioxidants has been shown previously [Jauslin et al., 2003] and

is reinforced here by the fact that ZYC-26, which has a phenolic ring, is cytoprotective against a BSO-induced insult while ZYC-23, which contains no such structure, is not (Fig. 4). Furthermore, PPT, which contains 3 phenol rings and has a significant protective effect at 10 nM and an EC₅₀ of 4.6 nM, is a more potent cytoprotectant when compared to 17 β -estradiol or ZYC-26, which contain only 1 and are less protective with EC₅₀ values between 15 and 23 nM (Figs. 3 and 5A). DPN, a compound with 2 phenol rings, was observed to have intermediate cytoprotective potency, with a EC₅₀ of 9.3 nM (Fig. 5B). In addition, although it is an ER α and ER β antagonist, ICI 182,780 has a protective effect at concentrations of 2 μ M (data not shown), likely due to the phenol ring in its structure (Fig. 1).

All of the phenolic compounds provided a statistically significant reduction in ROS in FRDA fibroblasts when given with 1 mM BSO, while compounds without phenol rings did not (Fig. 7). Moreover, the 3 phenol ring containing PPT (Fig. 1) is far more effective at reducing the levels of ROS than E2 or ZYC-26, which contain 1 phenol ring or DPN, which contains 2. This is consistent with the finding that PPT is a more potent cytoprotectant against BSO-induced oxidative stress. These observations provide a potential mechanism for the attenuation of the BSO effects seen with all phenolic steroids tested (Figs. 3-5).

These data support the hypothesis that estrogens protect against oxidative damage to the mitochondria in FRDA fibroblasts by direct antioxidant properties, rather than by stimulation of any known estrogen receptor, and that these antioxidant properties are dependent on the presence of at least one phenol ring in the molecular structure. Since there is a simple genetic test that can be done on the children of known FRDA allele

carriers, it is possible to determine the presence of Friedreich's ataxia in newborns, years before the cardio- and neurodegeneration and clinical symptoms begin, a time window during which estrogens and other antioxidants could potentially be clinically useful. This study presents the first data supporting estrogens and non-feminizing estrogens as a potential drug for the treatment and prevention of the symptoms of clinical Friedreich's ataxia.

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Figure legends

Figure 1. Structures of compounds assessed for protection against BSO toxicity in FRDA fibroblasts.

Figure 2. Effects of BSO on cell viability of FRDA fibroblasts. Depicted are mean \pm SD for n= 8 per group. * indicated $p<0.05$ versus vehicle control.

Figure 3. Effects of 17β -estradiol on cell viability in BSO-treated FRDA fibroblasts. Depicted are mean \pm SD for n= 8 per group. * indicated $p<0.05$ versus BSO alone-treated cells. ICI indicated ICI 182,780.

Figure 4. Effects of non-feminizing estrogens on cell viability in BSO-treated FRDA fibroblasts. Depicted are mean \pm SD for n= 8 per group. * indicated $p<0.05$ versus BSO alone-treated cells. ICI indicates ICI 182,780. ZYC-26 (A) indicates 2-adamantyl, 4 methyl estrone. ZYC-23 (B) indicates 2-adamantyl, 3-0-methyl estradiol.

Figure 5. Effects of an ER α preferring agonist, PPT (A), and an ER β preferring agonist DPN, (B) on cell viability in BSO-treated FRDA fibroblasts. Depicted are mean \pm SD for n= 8 per group. * indicated $p<0.05$ versus BSO alone-treated cells.

Figure 6. Effects of the membrane ER-preferring agonist, G1 (A), on cell viability in BSO-treated FRDA fibroblasts, and effects of the membrane ER-preferring antagonist,

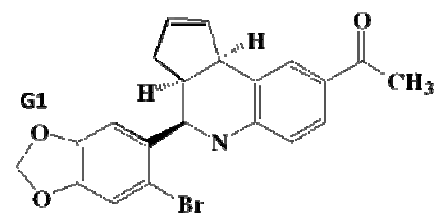
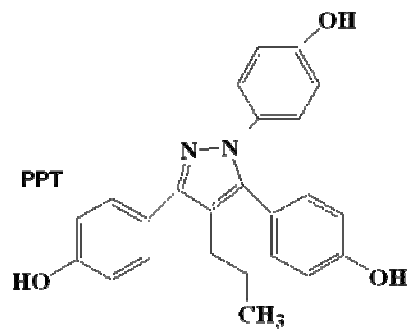
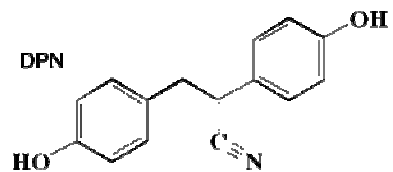
G15 (B), on E2-induced enhancement of cell viability in BSO-treated FRDA fibroblasts. Depicted are mean \pm SD for n= 8 per group. * indicated $p<0.05$ versus BSO alone-treated cells.

Figure 7. Effects of E2, PPT, DPN, ZYC-26 and ZYC-23 on the formation of ROS in BSO-treated FRDA fibroblasts. All steroid concentrations were 100nM. Depicted are mean \pm SD for n= 8 per group. * indicated $p<0.05$ versus BSO alone-treated cells. † indicated $p<0.05$ versus BSO + E2/DPN/ZYC-26 treated cell groups and $p<0.05$ versus DMSO vehicle control treated cells.

Figure 8. Calcein AM stained cells visually showing the effects of E2, ZYC-26, ZYC-23, PPT, DPN, G1 and G15 on cell viability in BSO-treated FRDA fibroblasts. BSO concentration was 1mM and all steroid concentrations were 100nM. Scale bar = 200 μ m.

Estrogen Receptor Agonists

17 β -Estradiol



ICI 178,820

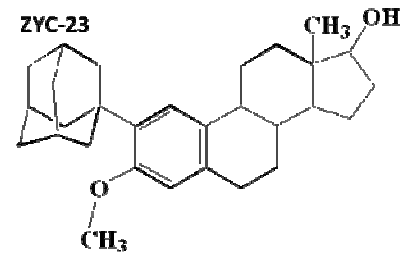
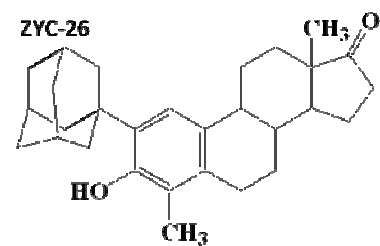
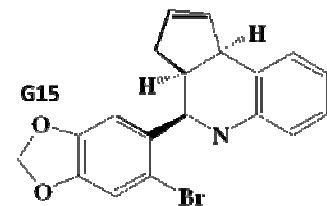
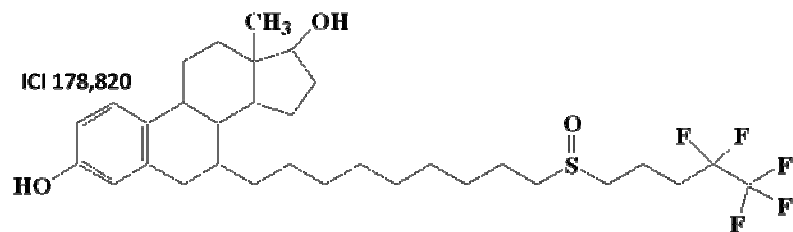


Figure 2

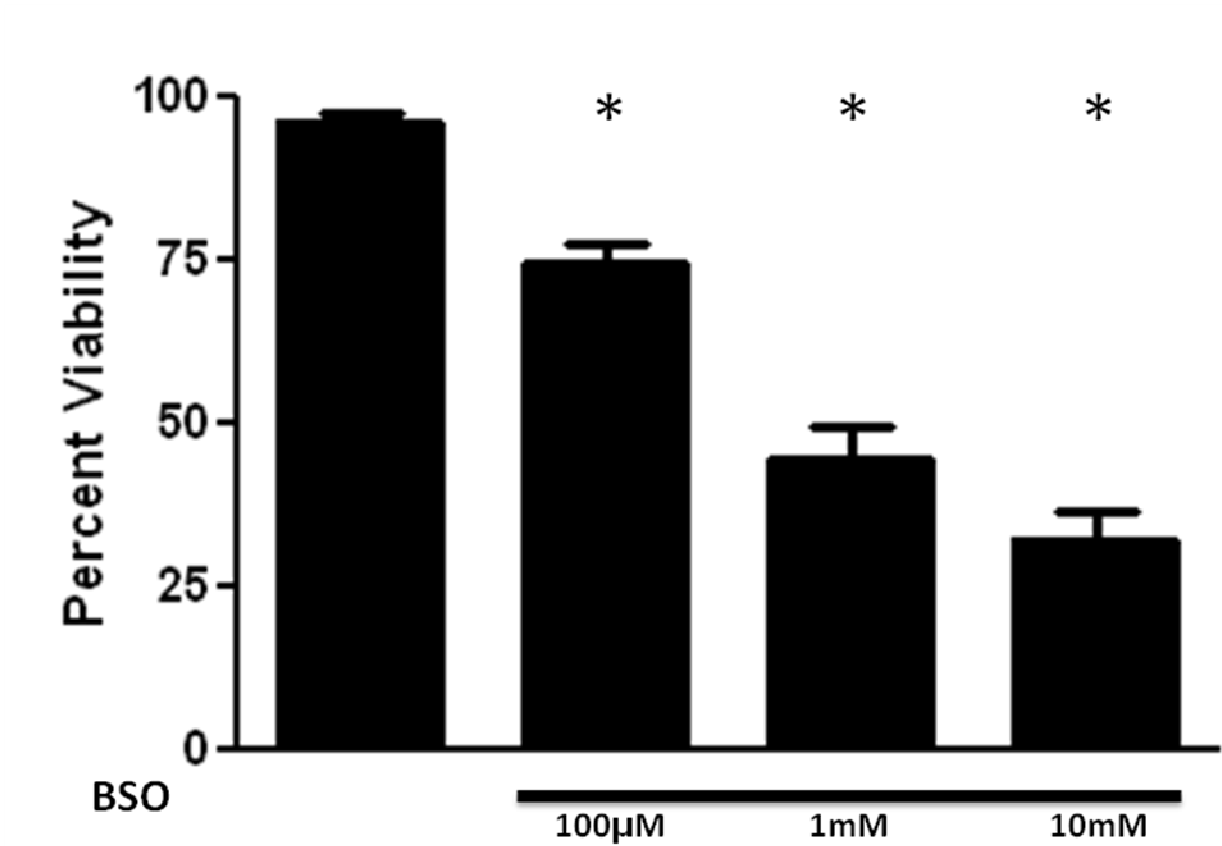


Figure 3

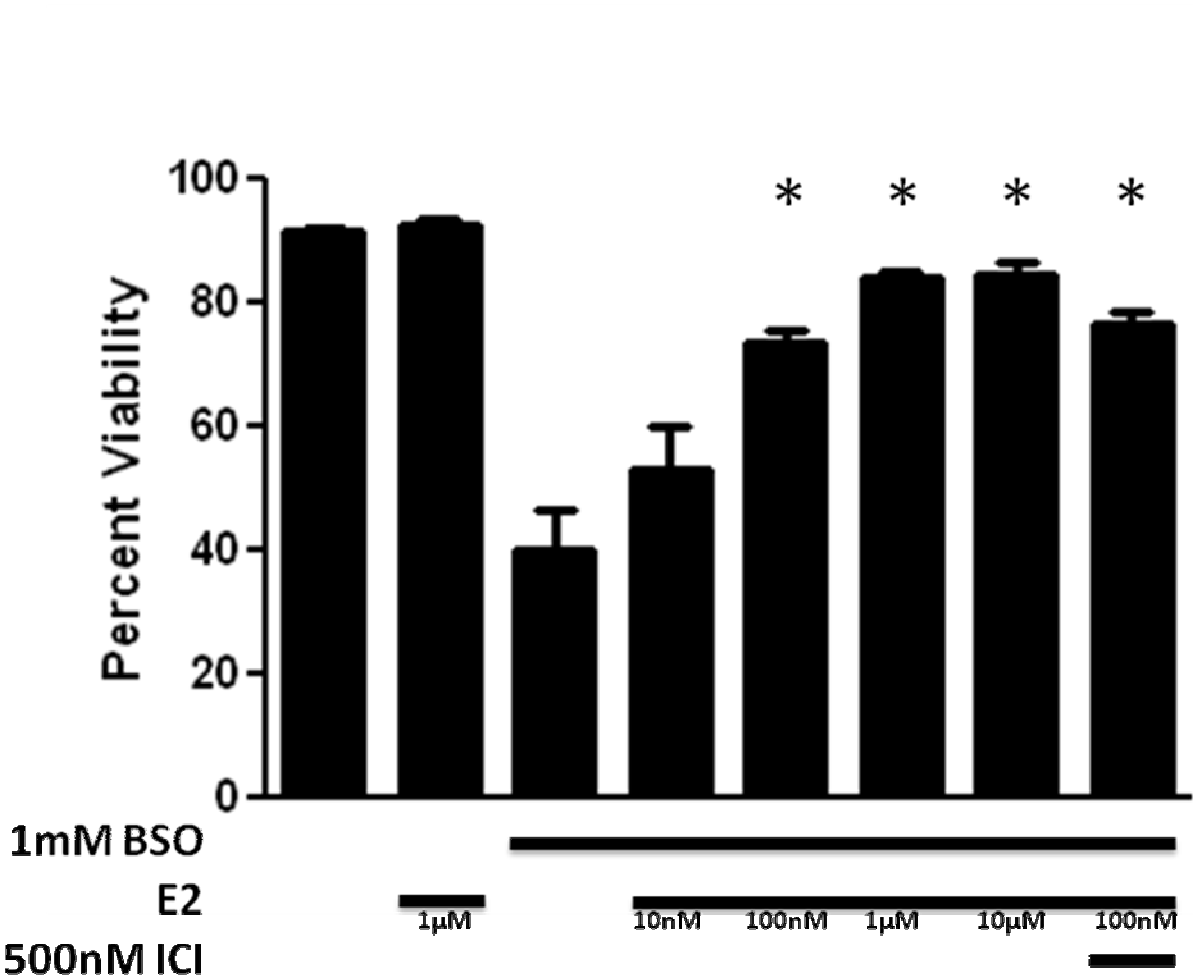


Figure 4

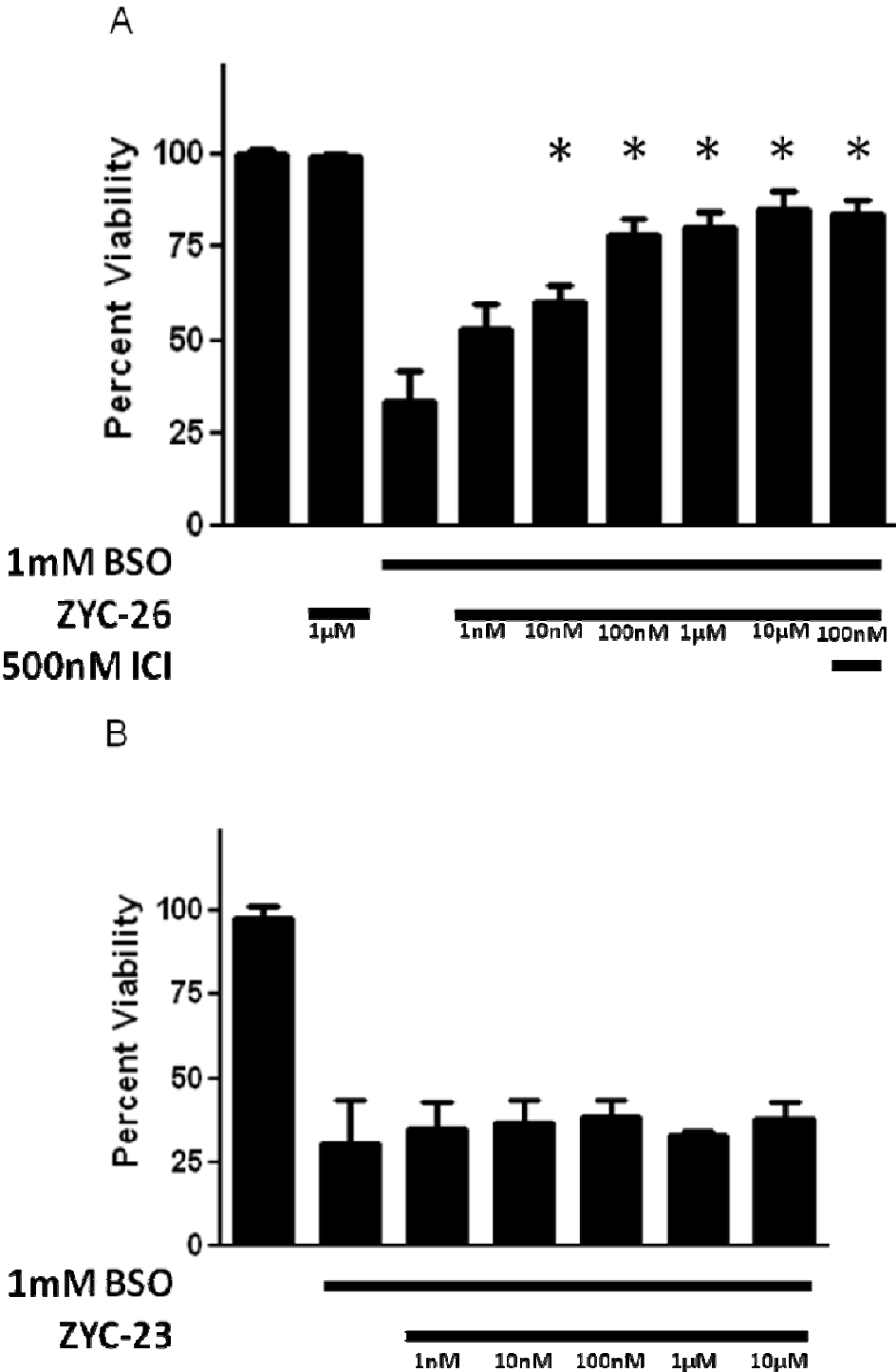


Figure 5

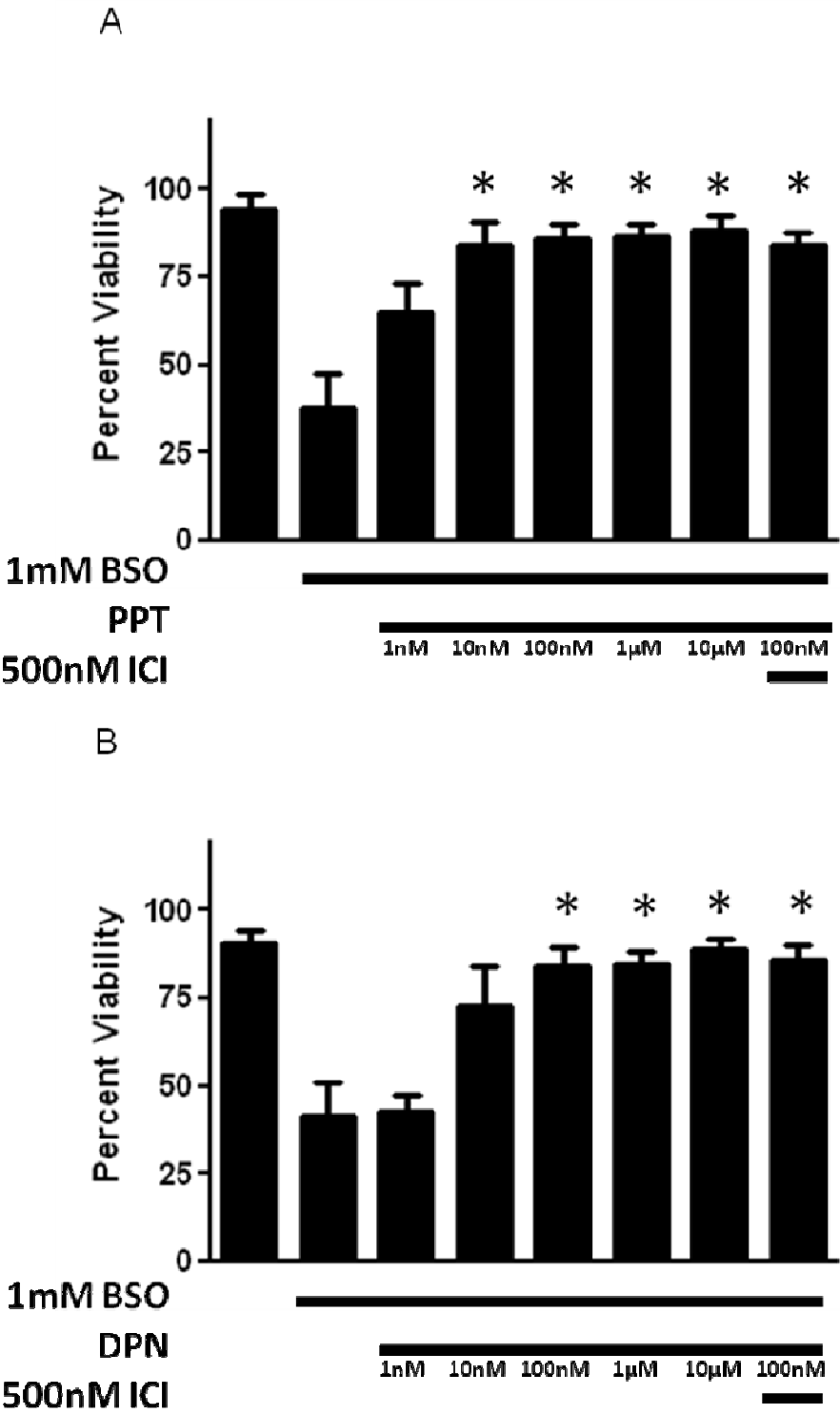


Figure 6

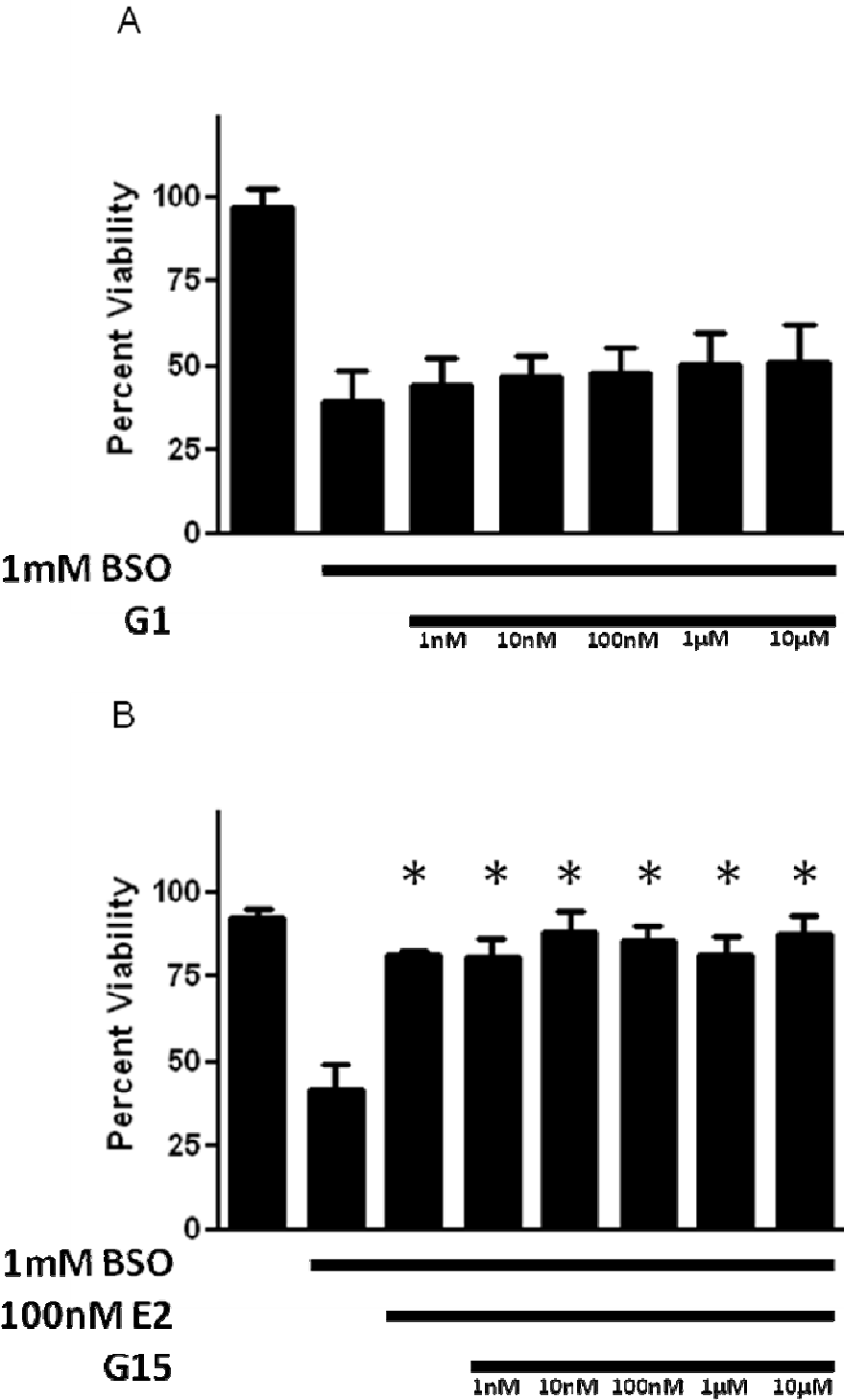


Figure 7

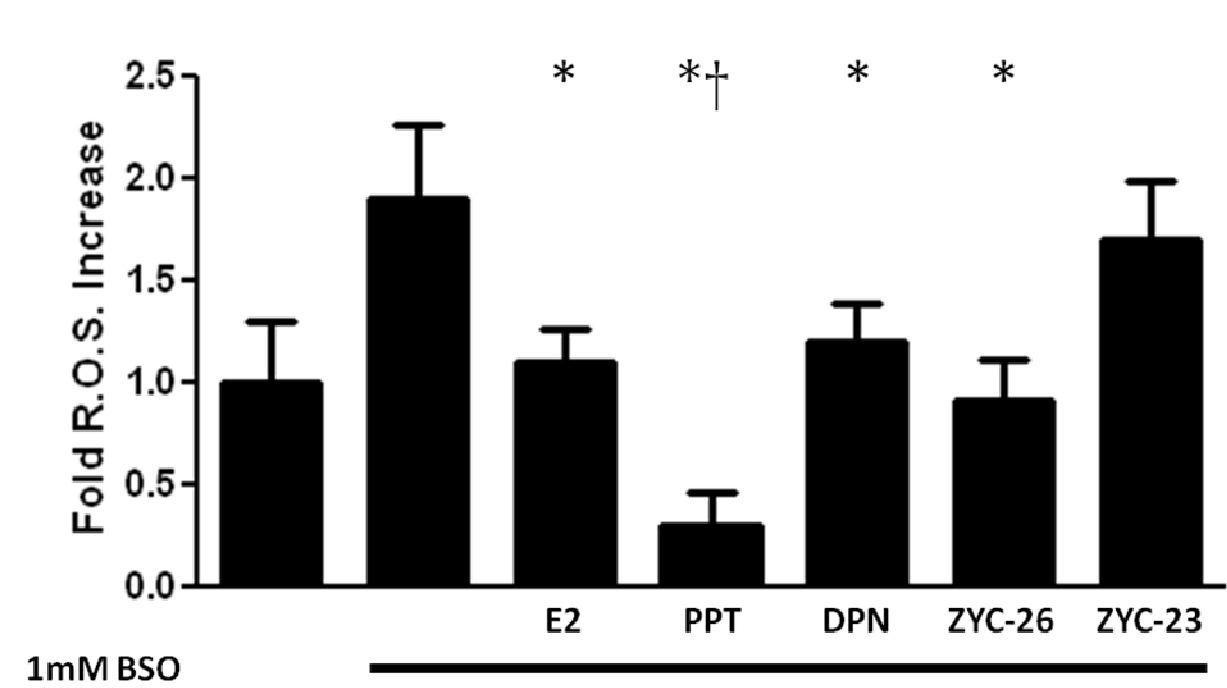
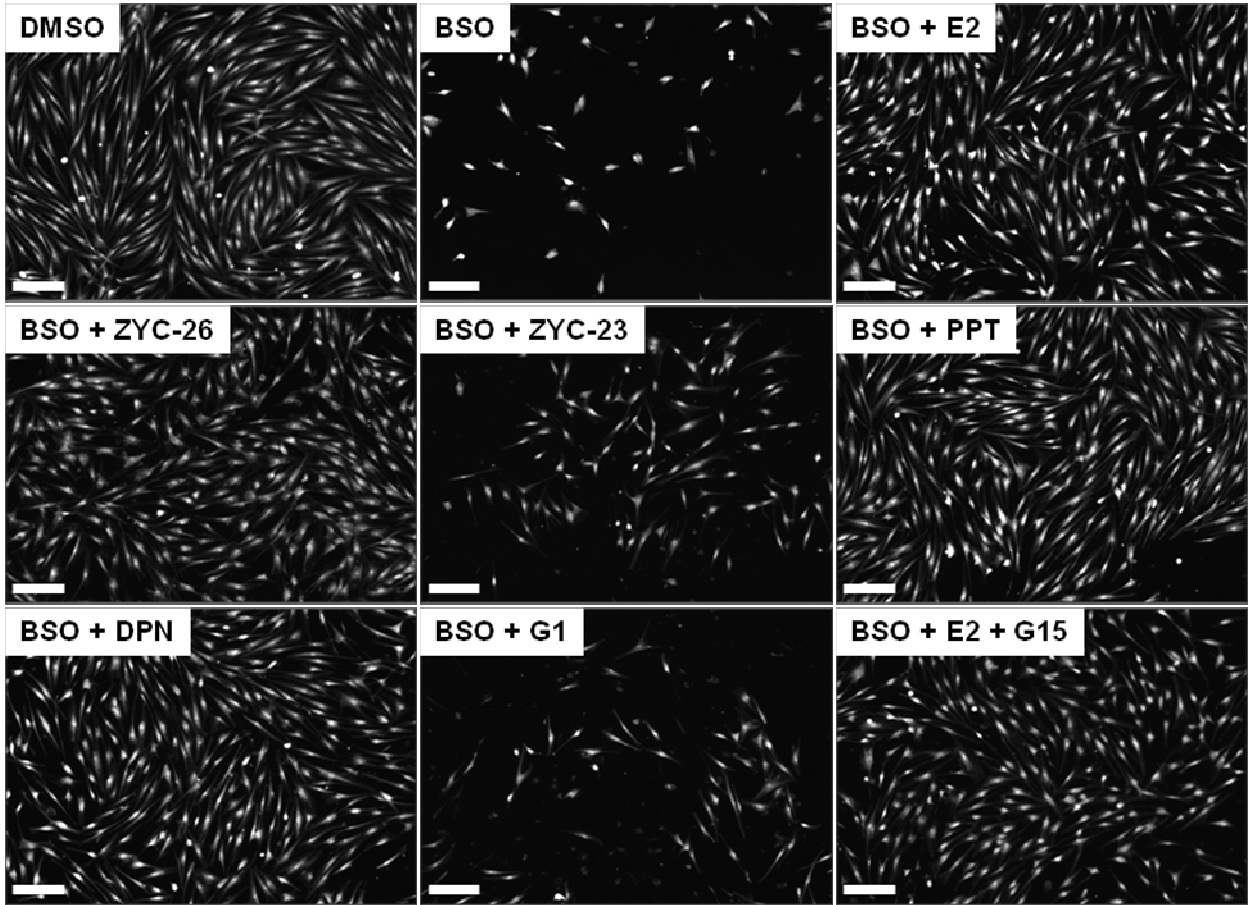


Figure 8



PREFACE TO CHAPTER III

In the previous chapter, we determined that estrogen-like compounds are able to protect Friedreich's ataxia fibroblasts from a BSO-induced oxidative insult independently of any known ER, provided that their structure contains at least one phenol ring. Here we provide more evidence for the ER-independent nature of this phenomenon using R- and S-Equol, enantiomers containing two phenol rings, with only the S-form having significant binding ability at ER β , the only ER present in FRDA fibroblasts

CHAPTER III

R- and S-Equol Have Equivalent Cytoprotective Effects in Friedreich's Ataxia

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Key Words: equol, 17 β -estradiol, antioxidants, Friedreich's Ataxia, fibroblasts, neuroprotection

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In preparation for publication

Abstract

Estradiol (E2) is a very potent cytoprotectant against a wide variety of cellular insults in numerous different cell models, including a Friedreich's ataxia (FRDA) model. Previously, we demonstrated that estrogen-like compounds are able to prevent cell death in an FRDA model seemingly independent of any known estrogen receptor (ER) by reducing reactive oxygen species (ROS) and the detrimental downstream effects of ROS buildup including oxidative damage to proteins and lipids and impaired mitochondrial function. Here we support the finding that this effect of E2 is ER-independent by first demonstrating that our cell model lacks ER α and expresses only low levels of ER β , then evaluating the effects of the potent soy-derived ER β agonist S-equol and its significantly less potent enantiomer, R-equol. Our results demonstrate that these biphenolic compounds, while significantly less potent and efficacious than E2, provide statistically similar attenuation of ROS and cytoprotection against an oxidative insult caused by L-buthionine (S,R)-sulfoximine (BSO). These data confirm that the protection seen previously with E2 was indeed unrelated to ER binding.

Introduction

First recognized in 1863 [Freidreich, 1863a, 1963b, 1963c], Friedreich's Ataxia (FRDA) is a hereditary ataxia characterized by an autosomal recessive GAA trinucleotide repeat in the *FXN* gene, resulting in the absence of frataxin protein [Harding, 1983; Campuzano et al., 1996]. Without sufficient levels of frataxin, cells are unable to maintain function of Fe-S cluster proteins essential for mitochondrial respiration and their cellular redox state leading to mitochondrial dysfunction, insufficient energy production and ultimately cell death, beginning in organs with greater energy requirements, such as the heart, brain and spinal cord [for review see: Santos et al., 2010].

First detected in humans in 1982 [Axelson et al., 1982], equol is a biphenolic isoflavone metabolized from the soy product daidzein by intestinal flora [Wang et al., 2005; Price and Fenwick, 1985; Kelly et al., 1993] in 14-59% of the human population [Akaza et al., 2004]. Equol is known to act as an antioxidant [Pereboom et al., 1999; Mitchell et al., 1998], decreases circulating estrogens and androgens [Duncan et al., 2000], inhibits DHT binding to its receptor [Lund et al., 2004] and decreases risks of prostate [Lund et al., 2004; Azaka et al., 2004; Mitchell et al., 2000] and breast cancer [Frankenfelt et al., 2004]. Separation of racemic equol mixtures shows that S-equol binds with very high affinity to ER β , while its enantiomer, R-equol has a far lower affinity for ER β , instead showing a preference for ER α [Muthyala et al., 2004; Setchell et al., 2005]. These enantiomers allow for the discrimination between effects due to antioxidant effects and those due to ER β activation.

We have previously shown that phenolic estrogens are able to prevent BSO-induced FRDA skin fibroblast death, as well as block the formation of ROS [Richardson et al., 2011], prevent lipid peroxidation, protein damage, depletion of ATP and support the mitochondria and oxidative phosphorylation [Richardson et al., 2012]. In the present study, we provide further evidence that E2 acts by an ER α - and ER β -independent mechanism. We demonstrate a lack of ER α and a very low level of ER β in FRDA fibroblasts by western blot. In addition, we show pharmacologically that ER β is not contributing to this process, as R- and S-equol have statistically equivalent efficacies and potencies, represented here as EC₅₀ values. These data indicate that it is the phenolic ring present in the compound structure of equol and E2 and not intrinsic receptor binding ability that is responsible for cytoprotective effects in this FRDA cell model. Although these compounds are substantially less efficacious and potent than compounds previously used [Richardson et al., 2011], this pharmacologic model lends support to the non-receptor mediated, non-genomic antioxidant mechanism of E2.

Methods

Cell Culture. Fibroblasts from a 30 year old Friedreich's Ataxia (FRDA) patient (Coriell Institute, Camden NJ, USA) were maintained in Dulbecco's Modified Eagle Medium (DMEM; ThermoScientific, Waltham, MA, USA) with 10% charcoal-stripped fetal bovine serum (CSFBS; ThermoScientific, Waltham, MA, USA), 1% GlutaMAX (ThermoScientific, Waltham, MA, USA) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C in 90% humidity and 5% CO₂. At the time of treatment, the FRDA fibroblast media was changed to phenol red- and sodium pyruvate-free DMEM (ThermoScientific, Waltham, MA, USA) and 1% penicillin-streptomycin. Experiments were conducted using cell passages 15-19.

Chemicals & Reagents. 17 β -Estradiol (E2) was acquired from Steraloids, Inc. (Newport, RI, USA). L-buthionine (S,R)-sulfoximine (BSO) was obtained from Sigma-Aldrich (St Louis, MO, USA). R- and S- Equol were obtained from the laboratory of Dr Robert J Handa at The University of Arizona.

Treatment Paradigm. FRDA fibroblasts were removed from culture with 0.25% Trypsin-EDTA (Invitrogen, Carlsbad, CA, USA) and plated on 96-well plates at a density of 3,000 cells per well in DMEM with 10% CSFBS, 1% GlutaMAX and 1% penicillin-streptomycin. After 24 hours the media was removed and replaced with phenol red- and sodium pyruvate-free DMEM with 1% penicillin-streptomycin. The cells were then treated for 12 to 48 hours with either dimethyl sulfoxide vehicle control (DMSO; Sigma-Aldrich, St Louis, MO, USA) or 1mM BSO in the presence of E2, R-equol or S-equol

((3S)-3-(4-Hydroxyphenyl)-7-chroman-6-ol). This duration of exposure was chosen based on our observation of BSO-induced enhancement of ROS at 12 hours and cell death at 48 hours [Richardson et al., 2011].

Calcein AM Cell Viability Assay. Cells were plated on a 96-well plate at a density of 5,000 cells per well, then treated with vehicle or 1mM BSO. After 48 hours of BSO treatment, the media was removed, and 1 µg/mL Calcein AM (CalBiochem, San Diego, CA, USA) in phosphate buffer pH 7.2 (PBS; Fisher Scientific, Pittsburg, PA, USA) was added to each well and the plate was incubated for 10 minutes at 37°C. Cell viability was determined with a Tecan Infinite M200 (Tecan Systems, Inc., San Jose, CA) plate reader with an excitation of 490nm and emission of 520nm at 48 hours.

Reactive Oxygen Species Assay. After 12 hours of treatment the media was removed from each well of the 96-well plate, and 100 µL of a 1 µM 2',7'-Dichlorodihydrofluorescein diacetate (DCFDA; AnaSpec Inc., Fremont, CA, USA) in PBS was added to each well. The plates were returned to a 37°C incubator for 20 minutes, then each well was washed three times with PBS and the resulting reaction was read on a Tecan Infinite M200 plate reader with an absorbance of 495 nm and an emission of 529 nm.

Data and Statistics. All data are displayed as mean \pm 1 standard deviation. These data were analyzed using the ANOVA against an alpha level of 0.05. All bar graphs were made using GraphPad Prism 5 and EC₅₀ calculations were made with GraphPad Prism 5.

For all groups, $n=8$ wells and experiments were repeated three times to ensure consistency.

Results

The effects of R- and S-equol on cell viability in BSO-treated FRDA fibroblasts. To determine the effect of R- and S-equol on cell viability, we first assessed their protective potential compared to 17 β -estradiol (E2) at 100 nM, a concentration previously shown to be very protective in this cell model [Richardson et al., 2011]. At 100 nM, both R- and S-equol provided statistically significant protection compared to the BSO-alone treated group, however the two groups did not differ significantly from each other (Fig. 2a). E2 also provided significantly more protection than either of these two compounds (Fig. 2a). A dose-response assessment showed that R- and S-equol have almost identical cytoprotective profiles at all concentrations (Fig. 2b), and EC₅₀ evaluation demonstrated that the two have statistically equivalent EC₅₀ values (Table 1), indicating that the cytoprotective effect is not due to stimulation of ER β .

The effects of R- and S-equol on BSO-induced reactive oxygen species (ROS)

formation. To determine the effects of R- and S-equol on ROS attenuation, these two compounds were again compared to E2 (Fig. 3a). BSO induced a 2-fold increase of ROS, which was prevented by 100 nM concentrations of E2, R-equol and S-equol. None of these groups differed from each other. In addition, a dose response curve for R- and S-equol shows that there is no significant difference in the ROS attenuation profiles of these two compounds at any concentration (Fig. 3b), and the EC₅₀ values do not differ significantly (Table 1).

Discussion

FRDA is the most common of the inherited ataxias world wide, affecting an estimated 1:50,000 to 1:20,000 people [Harding et al., 1983; Santos et al., 2010]. With the effective loss of functional frataxin throughout all organ systems, and the resulting ROS proliferation and mitochondrial respiration impairment, cells in organs most dependent on ATP production begin to degenerate [Marmolino, 2011; Santos et al., 2010]. This results in the loss of cells in the posterior columns and spinocerebellar tracts of the spinal cord, resulting in tremor and ataxia, as well as lateral and kyphoscoliosis, weakness, speech problems, pes cavitus, an increased incidence of diabetes mellitus and glucose intolerance and cardiac disorders, such as hypertrophic cardiomyopathy with interstitial fibrosis [Santos et al., 2010]. Disease onset and severity is variable depending on the number of GAA trinucleotide repeats present in the first intron of the *FXN* gene, although this alone is not able to account for the full course of the disease process [Klopstock et al., 1999]. A study of an Italian population has suggested that females may have less severe symptoms and a later onset when matched with males that have similar numbers of repeats, possibly indicating a hormonal role [Leone et al., 1990].

Estrogen and non-feminizing estrogens have been shown to be potently cytoprotective in many different cell and animal models of disease states [Behl, 2002; Simpkins, et al., 1997], including a FRDA cell model [Richardson et al., 2011]. Previous observations have demonstrated that antioxidants, especially mitochondrially targeted antioxidants [Jauslin et al., 2002; Jauslin et al., 2003], including estrogen receptor agonists and non-feminizing estrogens [Richardson et al., 2011] are protective against

FRDA. These effects have been shown to be ER independent and are instead based in the antioxidant properties of phenolic estrogens [Prokai et al., 2003; Prokai et al., 2006].

Equol is a naturally derived biphenolic (Fig. 1) product of soy digestion in a substantial percentage of the American population [Akaza et al., 2004]. It is created by intestinal flora as a racemic mixture of the R- and S-forms, with the S-form being very selective for ER β , the only ER present in FRDA fibroblasts [Richardson et al., 2012] while the R-form is only a very weak agonist at this receptor [Muthyala et al., 2004; Setchell et al., 2005]. Our results indicate that, while not as potent or efficacious as E2 (Fig. 2a and 3a) [Richardson et al., 2011], the R- and S-forms of equol are equally effective in attenuating ROS (Fig. 3b, Table 1) and preventing cell death (Fig. 2b, Table 1). These data indicate that equol, specifically the non-feminizing R-equol, could potentially be used to prevent or delay cell death and pathologic symptoms in FRDA and supports our previous hypothesis that estrogen-like compounds are acting in a manner unrelated to any known ER [Richardson et al., 2011; Richardson et al., 2012].

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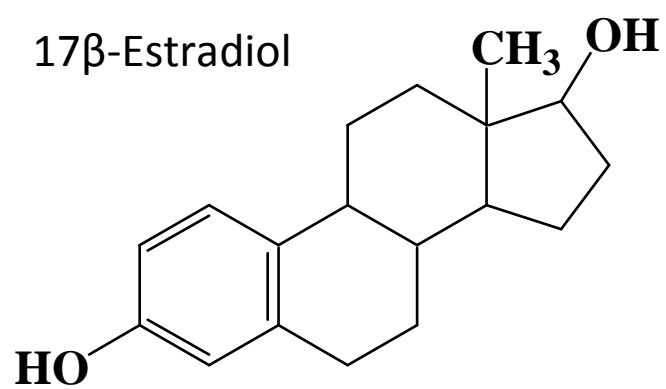
Figure legends

Figure 1. Structures of compounds assessed for protection against BSO toxicity in FRDA fibroblasts.

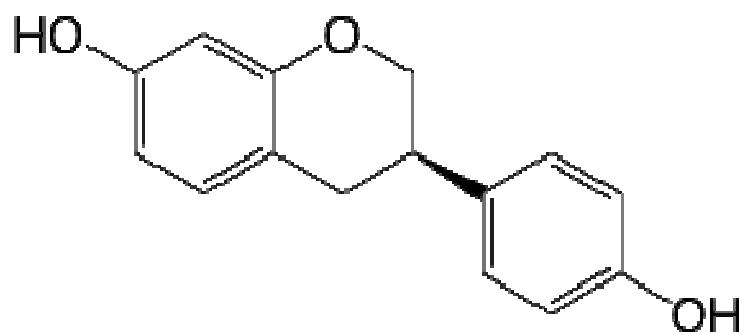
Figure 2. A.) Effects of 17 β -estradiol, R-equol and S-equol on cell viability in BSO-treated FRDA fibroblasts at 100nM. B.) Effects of R-equol and S-equol on cell viability in BSO-treated FRDA fibroblasts. Depicted are mean \pm SD for n= 8 per group. * indicated p<0.05 versus BSO alone-treated cells. † indicated p<0.05 versus BSO + R- or S-equol.

Figure 3. A.) Effects of 17 β -estradiol, R-equol and S-equol on ROS accumulation in BSO-treated FRDA fibroblasts at 100nM. B.) Effects of R-equol and S-equol on ROS accumulation in BSO-treated FRDA fibroblasts. Depicted are mean \pm SD for n= 8 per group. * indicated p<0.05 versus BSO alone-treated cells.

Figure 1



R-Equol



S-Equol

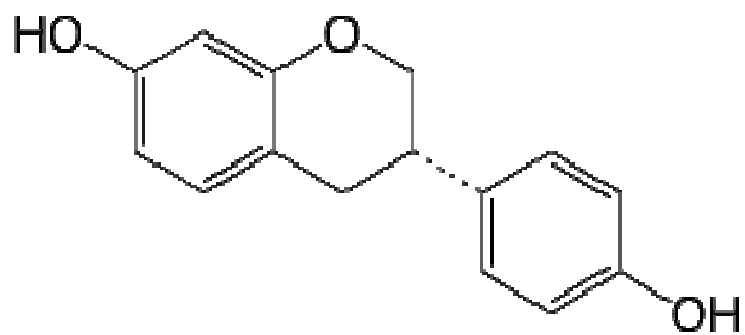


Figure 2

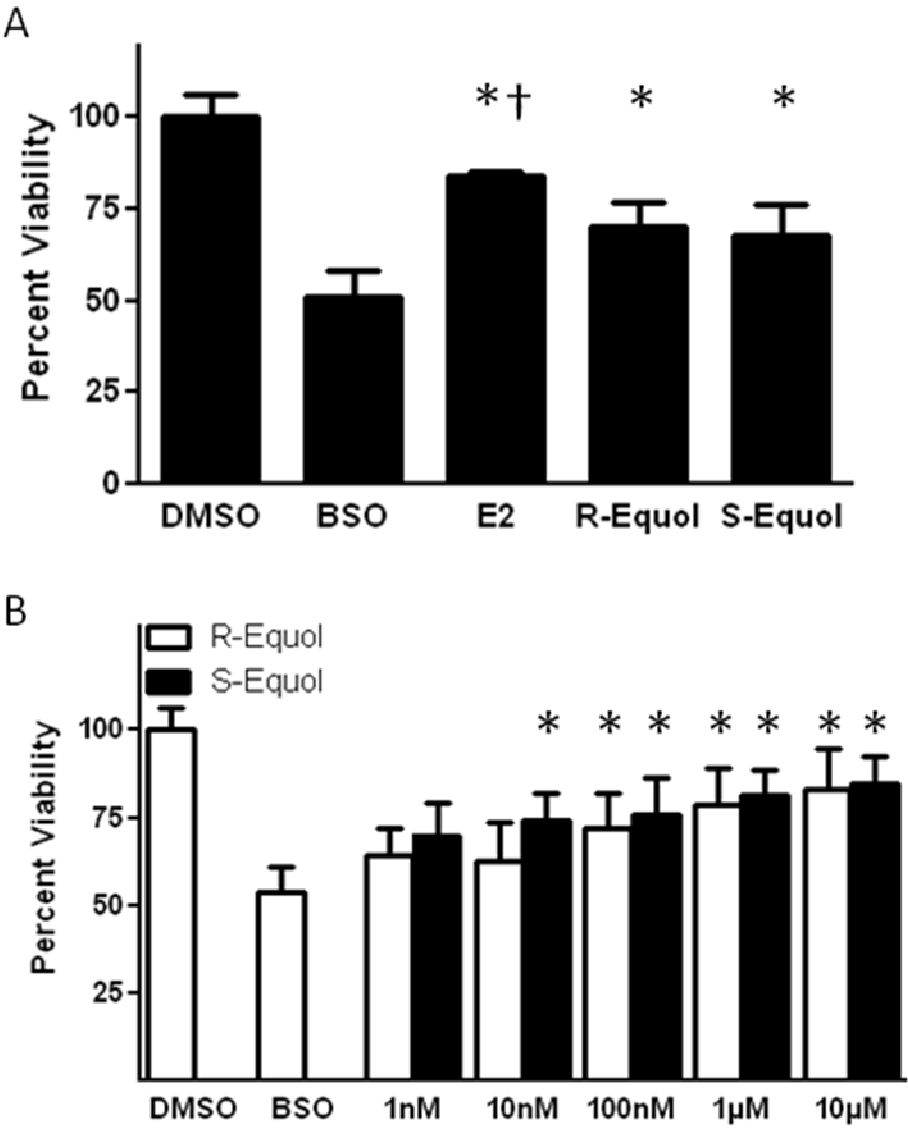


Figure 3

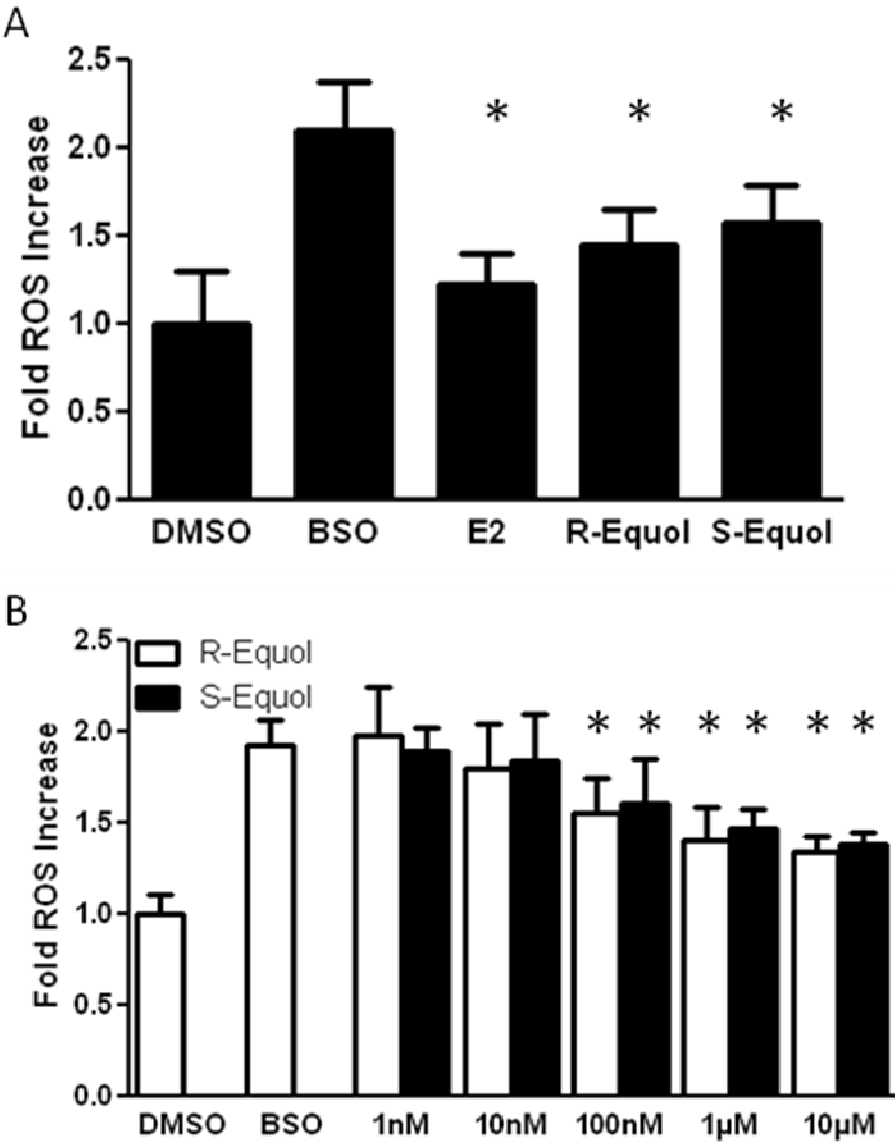


Table 1. EC₅₀ values for R- and S-equol with respect to cell viability and ROS attenuation.

Cell Viability

Compound	EC50 (nM)	Standard Error (nM)
R-Equol	440.5	21.11
S-Equol	459.9	12.75

Reactive Oxygen Species

Compound	EC50 (nM)	Standard Error (nM)
R-Equol	413.9	34.91
S-Equol	439.1	33.77

PREFACE TO CHAPTER IV

Having shown conclusively that estrogens protect this cell type from oxidative insult by a non-ER mediated mechanism in chapters II and III, we investigated the mechanism of the estrogen action by determining the sequelae of events beginning with BSO treatment and ending with cell death 48 hours later. Here we illustrate that estrogen cytoprotection is due to attenuation of reactive oxygen species and prevention of subsequent oxidative damage to mitochondrial components.

CHAPTER IV

Estrogen Prevents Oxidative Damage to the Mitochondria in Friedreich's Ataxia Skin Fibroblasts

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Abstract

Estrogen and estrogen-related compounds have been shown to have very potent cytoprotective properties in a wide range of disease models, including an *in vitro* model of Friedreich's ataxia (FRDA). This study describes a potential estrogen receptor (ER)-independent mechanism by which estrogens act to protect human FRDA skin fibroblasts from a BSO-induced oxidative insult resulting from inhibition of *de novo* glutathione (GSH) synthesis. We demonstrate that phenolic estrogens, independent of any known ER, are able to prevent lipid peroxidation and mitochondrial membrane potential ($\Delta\Psi_m$) collapse, maintain ATP at near control levels, increase oxidative phosphorylation and maintain activity of aconitase. Estrogens did not, however, prevent BSO from depleting GSH or induce increased production of GSH. The cytoprotective effects of estrogen appear to be due to a direct overall reduction in oxidative damage to the mitochondria, enabling the FRDA fibroblast mitochondria to generate sufficient ATP for energy requirements and better survive oxidative stress. These data support the hypothesis that phenol ring containing estrogens are possible candidate drugs for the delay and/or prevention of FRDA symptoms.

Introduction

First reported in 1863 by Nikolaus Friedreich [Friedreich, 1863a, 1863b, 1863c], Friedreich's Ataxia (FRDA) has an incidence of 1:50,000-1:20,000 and a carrier rate of 1:120-1:60 in the Caucasian population of the United States, making it the most prevalent form of hereditary ataxia [Delatycki et al., 2000; Harding, 1983; Harding and Zilkha, 1981; Lodi et al., 2006]. This disorder is inherited in an autosomal recessive manner caused by a GAA repeat expansion in the first intron of the FXN gene on chromosome 9q13-21 [Fujita et al., 1989; Hanauer et al., 1990], causing a self-associating complex of sticky DNA to form, hindering transcription [Sakamoto et al., 2001] and significantly reducing the expression of frataxin [Al-Mahdawi et al., 2006; Campuzano et al., 1996; Montermini et al., 1997; Santos et al., 2010]. The number of GAA repeats on the smaller allele is inversely proportional to the intracellular levels of frataxin [Campuzano et al., 1997] and positively correlated to the severity of patient symptoms [Dürr et al., 1996; Isnard et al., 1997]. Although the exact role of frataxin is currently unclear, its loss has two direct effects in several reported tissue types: impaired formation of iron-sulfur (Fe-S) clusters and a rise in intracellular reactive oxygen species (ROS) [Delatycki et al., 2000; Gakh et al., 2006; Lodi et al., 2006; Santos et al., 2010]. The decrease in Fe-S containing proteins, such as heme, electron transport chain (ETC) complexes I-III and the Krebs's cycle protein aconitase severely impairs cellular respiration [Bradley et al., 2000; Bulteau et al., 2004; Rötig et al., 1997], which is further complicated by simultaneous oxidative damage to these mitochondrial proteins [Al-Mahdawi et al., 2006; Bulteau et al., 2004; Chantrel-Groussard et al., 2001; Gakh et al., 2006; Prokai et al., 2007]. These events all culminate in an inability of the mitochondria to fulfill the cell's energy

requirements resulting in cell death [Santos et al., 2010], a mechanism of death common to many neurodegenerative diseases [for review see: Simpkins and Dykens, 2008; Simpkins et al., 2008].

First established more than a decade ago [Behl et al., 1995; Bishop and Simpkins, 1994], the neuro- and cytoprotective effects of 17 β -Estradiol (E2) are well known. However the exact mechanisms remain elusive [Simpkins and Dykens, 2008; Simpkins and Singh, 2008]. There are now numerous reports showing that estrogen and estrogen-like compounds are effective in protecting against a wide variety of insults in numerous different cell types [Behl, 2002], including human Friedreich's ataxia skin fibroblasts [Richardson et al., 2011]. In a previous study, we showed that several estrogen-like compounds are extremely potent and efficacious cytoprotectants of human FRDA fibroblasts against L-buthionine (S,R)-sulfoximine (BSO)-induced oxidative stress independent of any known estrogen receptor (ER) [Richardson et al., 2011]. This effect appears to be dependent on the presence of at least one phenol ring in the molecular structure of the compound and is at least in part due to antioxidant properties and the attenuation of reactive oxygen species [Richardson et al., 2011], a strategy previously investigated with other potential antioxidants [Jauslin et al., 2002, 2003, 2007; Lodi et al., 2006; Santos et al., 2010]. However, as in other cell and animal disease models, the precise mechanism of estrogen action in Friedreich's ataxia is not yet fully understood.

In this study, we investigate the mechanism of estrogen action in human FRDA skin fibroblasts. Using BSO to induce oxidative stress, we show that all phenolic estrogen-like compounds tested are able to attenuate ROS production [Richardson et al., 2011], prevent lipid peroxidation and maintain mitochondrial function. This occurs

without the prevention of BSO-induced reduction of glutathione (GSH). These effects are also independent of any known ER. These data presented here indicate that estrogens effectively prevent pro-oxidant stress in the mitochondria [Jauslin et al., 2002, 2003; Lodi et al., 2006] by preventing the excess ROS associated with FRDA from damaging mitochondrial enzymes and inducing cell death.

Methods

Cell Culture. Fibroblasts from a 30 year old FRDA patient, obtained from Coriell Institute (Camden, NJ, USA) were maintained in Dulbecco's Modified Eagle Medium (DMEM; ThermoScientific, Waltham, MA, USA) with 10% charcoal-stripped fetal bovine serum (FBS; ThermoScientific), 1% GlutaMAX (ThermoScientific) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C, 5% CO₂ and 90% humidity. Before vehicle or BSO treatment, FRDA fibroblast media was changed to phenol red- and sodium pyruvate-free DMEM (ThermoScientific) containing 1% penicillin-streptomycin. All experiments were conducted with FRDA cells from passage 14-21.

Chemicals & Reagents. 17 β -Estradiol (E2) was obtained from Steraloids, Inc. (Newport, RI, USA). L-buthionine (S,R)-sulfoximine (BSO) was acquired from Sigma-Aldrich (St Louis, MO, USA). ICI 182,780, 4,4',4'-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT) and diarylpropionitrile (DPN) were purchased from Tocris Bioscience (Ellisville, MO, USA). ZYC-26 and ZYC-23 were synthesized in the Covey laboratory [Perez et al., 2006]. Structures for these steroids were drawn using ChemDraw software (CambridgeSoft, Cambridge, MA), and are provided in Fig. 1.

Steroid Treatment. FRDA fibroblasts were plated in 24- or 96-well plates at a density of 3,000-35,000 cells per well in DMEM with 10% FBS, 1% GlutaMAX and 1% penicillin-streptomycin. After 24 hours the growth media was removed and replaced with the phenol red- and sodium pyruvate-free DMEM. The cells were then treated for 12-48

hours, depending on the assay, with either dimethyl sulfoxide vehicle control (DMSO; Sigma-Aldrich, St Louis, MO, USA) or 1mM BSO in the presence of 100nM E2, DPN, PPT, ZYC-26 or ZYC-23, concentrations of estrogen-like molecules which have been shown to be neuroprotective in various cell lines [Yi et al., 2008] and cytoprotective in this FRDA fibroblast line [Richardson et al., 2011].

Calcein AM Cell Imaging. Cells were plated on a 96-well plate at a density of 5,000 cells per well, then treated with vehicle or 1 mM BSO. After 24, 36 and 48 hours of BSO treatment, the media was removed, and 1 µg/mL Calcein AM (CalBiochem, San Diego, CA, USA) in phosphate buffer pH 7.2 (PBS; Fisher Scientific, Pittsburg, PA, USA) was added to each well and the plate was incubated for 10 minutes at 37°C. The cells were then photographed using a Zeiss Axio Observer Z1 inverted microscope (Carl Zeiss MicroImaging, Thornwood, NY).

Lactate Dehydrogenase (LDH) Cell Viability Assay. After 48 hours of treatment 50 µL of media was removed from each well of the 96-well plate and placed in a separate 96-well plate. A 100 µL solution consisting of 12 mL of 200 mM pH 8.2 Tris(hydroxymethyl)aminomethane hydrochloride (Sigma-Aldrich, St Louis, MO, USA) with 50 µL lactic acid and 4.2 mg iodonitrotetrazolium chloride (INT; Sigma-Aldrich, St Louis, MO, USA), 1.1 mg phenazine methosulphate (PMS; Sigma-Aldrich, St Louis, MO, USA) and 10.8 mg β-nicotinamide adenine dinucleotide hydrate (NAD; Sigma-Aldrich, St Louis, MO, USA) was added to each well. The absorbance of the resulting reaction was read with a Tecan Infinite M200 plate reader at 490 nm and recorded once

the reaction is linear for greater than 2 minutes. Cell viability for each well (WellX) was determined by: $100 - (100 * (\text{WellX} - \text{media}) / (0.1\% \text{ TritonX100} - \text{media}))$. Measurements were then confirmed by visual inspection of the FRDA fibroblasts.

Western Blots. FRDA fibroblasts and 661W photoreceptor cells were grown in 10 cm plates until ~80% confluent. The cells were then removed from the plates using rubber cell scrapers and sonicated in RIPA lysis buffer. A Lowry assay was run to determine protein concentration for normalization and 20 µg of protein was loaded into each western blot well. ERα and ERβ were detected using ERα (H-184) rabbit polyclonal IgG antibody and ERβ (H-150) rabbit polyclonal IgG antibody, obtained from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). GAPDH (6C5) mouse monoclonal IgG antibody, also obtained from Santa Cruz Biotechnology, was used as a control to ensure equivalent loading of protein loaded into each well.

Lipid Peroxidation Assay. Cells were plated on 10 cm dishes and grown until ~80% confluent. The cells were then treated with DMSO vehicle control or BSO and 100 nM E2, PPT, DPN, ZYC-26 or ZYC-23 in phenol red- and sodium pyruvate-free DMEM for 24 hours. At the end of treatment, the FRDA fibroblasts were removed from the plates using a rubber cell scraper and treated according to the Cayman 8-Isoprostane EIA protocol (Cayman Chemical Company, Ann Arbor, MI). The resulting absorbance was read on a Tecan Infinite F200 plate reader (Tecan Systems, Inc., San Jose, CA) at 340nm.

Aconitase Assay. Cells were plated in 10 cm dishes and grown until ~80% confluent. The cells were then treated with DMSO vehicle control or BSO and 100 nM E2, PPT, DPN, ZYC-26 or ZYC-23 in phenol red- and sodium pyruvate-free DMEM for 24 hours. At the end of treatment, the FRDA fibroblasts were removed from the plates using a rubber cell scraper and treated according to the Cayman protocol. The resulting absorbance was read on a Tecan Infinite F200 plate reader at 340 nm.

Mitochondrial Respiration Measurement. Human FRDA fibroblasts were plated in a 24-well Seahorse XF-24 assay plate at 35,000 cells/well and grown in FBS-containing DMEM media for 24 hours before being treated with either BSO or DMSO vehicle control and steroids for another 24 hours in FBS- and Phenol-red-free DMEM media. On the day of metabolic flux analysis, cells were changed to unbuffered DMEM media (DMEM base medium supplemented with 25 mM glucose, 10 mM sodium pyruvate, 31 mM NaCl, 2 mM GlutaMax, pH 7.4) and incubated at 37 °C in a non-CO₂ incubator for 1 hr. All media was adjusted to pH 7.4 on the day of assay. Eight baseline measurements of OCR and ECAR were taken before sequential injection of mitochondrial inhibitors, oligomycin (10 µM), FCCP (1 µM) and rotenone (5µM). Four measurements were taken after each addition of mitochondrial inhibitor before injection of the next inhibitor. Oxygen consumption rate (OCR, an indicator of oxidative phosphorylation) and extracellular acidification rate (ECAR, an indicator of glycolysis) were automatically calculated and recorded by the Seahorse XF-24 software (Seahorse Bioscience, North Billerica, MA, USA).

ATP Assay. Cells were plated on 10 cm dishes and grown until ~80% confluent. The cells were then treated with DMSO vehicle control or BSO and 100nM E2, PPT, DPN, ZYC-26 or ZYC-23 in phenol red- and sodium pyruvate-free DMEM for 24 hours. At the end of treatment, the FRDA fibroblasts were removed from the plates using a rubber cell scraper and treated according to the Abcam protocol (Abcam Inc., Cambridge, MA), then was read with an excitation of 535 nm and emission of 587 nm on a Tecan Infinite F200 plate reader.

Mitochondrial Membrane Potential ($\Delta\Psi_m$) Measurement. Cells were plated on 96-well plates at a density of 3,000 cells/well. The cells were then treated with DMSO vehicle control or BSO and 100 nM E2, PPT, DPN, ZYC-26 or ZYC-23 in phenol red- and sodium pyruvate-free DMEM for 36 hours. A fluorescence resonance energy transfer (FRET) assay was used in this study to measure mitochondrial membrane potential collapse. In this assay, nonyl acridine orange (NAO; Enzo Life Sciences Inc., Plymouth Meeting, PA, USA) was used to stain cardiolipin in the inner mitochondrial membrane. Tetramethylrhodamine, ethyl ester, perchlorate (TMRE; AnaSpec Inc., Fremont, CA, USA) was added simultaneously with NAO to the cell culture to quench the NAO fluorescent signal, and incubated for 20 minutes at 37°C in the dark. As mitochondrial membrane potential collapsed at 36 hours after BSO treatment, TMRE was released from the mitochondria, allowing the NAO fluorescent signature to be read with an excitation of 495nm and an emission of 519nm with a Tecan Infinite F200 plate reader [Dykens et al., 2002; Dykens and Stout, 2001; Wang et al., 2006].

Glutathione Assay. Cells were plated on 10 cm dishes and grown until ~80% confluent. The cells were then treated with DMSO vehicle control or BSO and 100 nM E2, PPT, DPN, ZYC-26 or ZYC-23 in phenol red- and sodium pyruvate-free DMEM for 24 hours. At the end of treatment, the FRDA fibroblasts were removed from the plates using a rubber cell scraper and treated according to the Cayman protocol. The resulting absorbance was read on a Tecan Infinite F200 plate reader at 410 nm.

Data and Statistics. All data are displayed as mean \pm 1 standard deviation. These data were analyzed using ANOVA against an alpha level of 0.05. All graphs were made using GraphPad Prism 5. For all groups, n=8 wells and experiments were repeated three times to ensure consistency.

Results

Timeline of events in BSO-induced FRDA fibroblast cell death. It was previously reported that after the application of 1 mM BSO, large-scale cell death occurs at 48 hours (Fig. 3a) [Jauslin et al., 2002; Richardson et al., 2011], with a peak ROS level at 12 hours [Richardson et al., 2011]. Here we have determined a timeline of events beginning with BSO treatment that lead to death in these FRDA fibroblasts. At 12 hours, ROS levels peak at 2-3 fold greater than control in an estrogen-preventable manner. This is followed by a rise in lipid peroxidation, impairment of aconitase activity, disruption in mitochondrial respiration and reduced ATP content at 24 hours (Figs. 4, 5, 6 and 7) and collapse of the mitochondrial membrane potential ($\Delta\Psi_m$) at 36 hours (Fig. 8) in estrogen-preventable manners. Finally, at 48 hours there is widespread cell death, which can be significantly reduced by phenolic estrogens (Fig. 3b) [Richardson et al., 2011].

Human FRDA fibroblasts express small amounts of ER β , but not ER α . To determine the presence or absence of ER α and ER β in human FRDA fibroblasts, western blots were run showing the intracellular presence of these proteins. Our western blots indicate the absence of ER α in human FRDA fibroblasts and the presence of very small amounts of ER β compared with 661W cells (Fig. 2). These data agree with our previous pharmacological observations utilizing ICI 182,780 and ZYC compounds, showing that estrogen is likely not acting through any known ER to produce its effects on cell viability and instead acts to block production of free radicals [Richardson et al., 2011].

Effects of steroids on cell viability in BSO-treated FRDA fibroblasts. It has been previously reported that after the application of 1 mM BSO, large scale death occurs at 48 hours (Fig. 3a) [Jauslin et al., 2002; Richardson et al., 2011], with peak ROS levels at 12 hours [29]. To determine the effect of 100 nM E2, PPT, DPN, ZYC-26 and ZYC-23 on BSO-treated fibroblasts, we measured cell viability via a LDH assay (Fig. 3b). BSO decreased cell viability from $96\pm1\%$ in DMSO-control cells to $47\pm7\%$. All of the phenolic steroids provided significant protection at 100 nM, with E2 at $91\pm1\%$, PPT at $94\pm6\%$, DPN at $94\pm4\%$ and ZYC-26 at $89\pm5\%$. ZYC-23 had no significant effect at 100 nM with an average cell viability of $49\pm5\%$, again indicating that the phenolic ring is the crucial part of the estrogen molecule in providing protection in this cell type (Fig. 1).

Effects of estrogens on lipid peroxidation measurements. To determine the extent of lipid peroxidation produced by BSO-related oxidative stress, 8-isoprostane was measured at 24 hours, in DMSO control and BSO-treated cells in the presence and absence of E2, PPT, DPN, ZYC-26 and ZYC-23 (Fig. 4). BSO increased the level of lipid peroxidation by more than 1.5-fold over DMSO control cells, as measured by intracellular 8-isoprostane concentration at 24 hours. This increase in lipid peroxidation was prevented by each of the phenolic estrogens, but not ZYC-23, indicating that the antioxidant properties of the phenolic rings of these compounds are able to prevent not only ROS accumulation, but also the resulting oxidative damage to intracellular molecules in these cells.

Effects of estrogens on aconitase activity. Aconitase, an enzyme in the Krebs cycle responsible for conversion of citrate to isocitrate, is an iron-sulfur containing protein extremely sensitive to reactive oxygen species and has been shown to be significantly inhibited in human FRDA cardiomyocytes, although not in other tissues [Lodi et al., 2006]. Here, we show that in human FRDA fibroblasts there was more than a 50% decrease in aconitase activity in cells treated with 1 mM BSO, and this was partially prevented with co-treatment of all tested phenolic estrogens, but not ZYC-23 (Fig. 5). There was a trend toward compounds with increased numbers of phenolic rings having an increased protective effect in terms of aconitase activity levels. This suggests that estrogens are preventing the loss of aconitase activity by preventing ROS damage to this enzyme.

Effects of estrogens on mitochondrial respiration. To determine if BSO decreases the level of aerobic ATP production, we assessed the oxygen consumption rate (OCR) with a Seahorse XF-24 metabolic flux analysis (Fig. 6a). BSO-induced oxidative stress significantly decreased both the basal respiratory rate (Fig. 6b) and the maximal respiratory rate (Fig. 6c) suggesting that at 24 hours of BSO treatment there was already permanent damage inflicted on the mitochondria. Interestingly, in the BSO-treated cells, the maximal respiratory rate (45.4 ± 1.0 pMoles/min) was significantly lower than the basal respiratory rate in the DMSO vehicle control cells (50.1 ± 2.0 pMoles/min) (Fig. 6a). Both E2 and ZYC-26 were able to partially offset the decreased respiratory capacity in both basal and maximal states (Fig 6a). These two estrogens are statistically equivalent in terms of basal respiratory rate rescue, however ZYC-26 appears to be less efficacious at

preventing mitochondrial impairment under the maximal state (Figs. 6b and 6c). In all measurements, ZYC-23 provided no significant increase in OCR compared to BSO-alone treated cells. Extracellular acidification rate (ECAR), a measure of anaerobic glycolysis was not statistically altered in any of the four groups (data not shown). Importantly, all of the differences noted in Figures 6a-6c can be attributed solely to mitochondrial dysfunction in living FRDA fibroblasts, as there was no significant cell death present at 24 hours (Fig. 3a). These data indicate that phenol ring containing estrogens are able to partially prevent BSO-induced free radical damage to the mitochondria, independent of any known ER, while non-phenol ring containing estrogens are not.

Effects of estrogens on cellular ATP concentration. To determine the effect of phenolic estrogens on ATP production, we evaluated each of these estrogen-like compounds with an intracellular ATP concentration assay. Although ATP production and concentration in FRDA fibroblasts is relatively low under baseline conditions compared to other cell types such as primary neurons, BSO significantly reduced the intracellular ATP content by about 35% beyond this level, an effect that was partially prevented by the phenolic estrogens E2, PPT, DPN and ZYC-26, but not the non-phenolic ZYC-23 (Fig. 7).

Mitochondrial Membrane Potential ($\Delta\Psi_m$) Collapse. To determine the effect of estrogens on mitochondrial membrane potential collapse, an event following ROS-induced mitochondrial damage and preceding cell death, we assessed each of our phenolic and non-phenolic compounds with a FRET assay at 36 hours after BSO

treatment, a time point at which there was a peak rise in NAO fluorescence in the BSO treated cells (data not shown). At 36 hours, before significant cell death occurs (Fig. 3a), there was a 4-5 fold increase in NAO fluorescence in BSO treated cells compared with DMSO controls. This effect was prevented by the addition of 100 nM of the phenolic estrogens E2, PPT, DPN and ZYC-26, but not ZYC-23 (Fig. 8). These data indicate that estrogen-like compounds are able to prevent oxidative-stress induced collapse of $\Delta\Psi_m$, an event indicating mitochondrial function disruption that occurs prior to cell death (Fig. 3a), and that this effect is dependent on the presence of a phenol ring in the molecular structure.

Effects of estrogens on glutathione concentrations. To determine if estrogens were acting to prevent BSO-induced declines in GSH, we assessed GSH concentrations in control cells and those treated with BSO for 24 hours. BSO significantly reduced the levels of GSH below baseline control, and none of the estrogen-like compounds assayed had any effect on GSH concentrations (data not shown). These data show that estrogens are not acting simply to prevent BSO from depleting glutathione or to induce glutathione synthesis *in vitro*.

Discussion

The potential for estrogens to protect a number of different cell and tissue types against a wide range of insults has been known for more than a decade [Behl, 2002; Bishop and Simpkins, 1994; Simpkins and Singh, 2008]. While clinical trials for estrogens have fallen short of expectations [Simpkins and Singh, 2008], there is still considerable evidence that estrogens have significant potential to act as neuroprotective agents in Alzheimer's disease, Parkinson's disease, ischemic stroke, traumatic brain injury and many other acute and chronic neurological states [Grandbois et al., 2000; Simpkins et al., 1997; Simpkins and Singh, 2008; Wigginton et al., 2010]. We have also previously reported that 17 β -estradiol and other estrogen-like compounds are able to significantly attenuate ROS production and prevent cell death in a human Friedreich's ataxia skin fibroblast model [Richardson et al., 2011]. While the exact mechanism of estrogen neuroprotection is currently unclear, there is mounting evidence that the protective effects of estrogen-related compounds may occur in cells in a nongenomic manner, independent of estrogen receptor activation and subsequent gene expression [Behl and Manthey, 2000; Behl and Moosmann, 2002; Prokai and Simpkins, 2007; Rupprecht and Holsboer, 1999; Simpkins and Dykens, 2008]. The antioxidant properties of estrogens [Prokai et al., 2006] are due to the presence of a phenol at position 3 on the A-ring of estrogens [Prokai et al., 2003; Prokai and Simpkins, 2007]. This phenol ring is responsible for attenuating ROS created by the Fenton reaction *in vitro*, produced in this study using BSO, by a cyclic phenol-quinol mechanism [Prokai et al., 2003].

FRDA is the most common inherited ataxia in the world, affecting at least 1:50,000 individuals in the United States [Harding, 1983]. Initiated by an autosomal

recessive GAA repeat expansion in the first intron of the FXN gene on chromosome 9 [Fujita et al., 1989; Hanauer et al., 1990], there is a depletion of frataxin protein in all of the affected individual's cells, resulting in severe impairment of mitochondrial respiration [Santos et al., 2010]. Classically, FRDA symptoms begin to appear in patients with >100 GAA repeat sections in the first intron of the FXN gene, with the number of repeats on the smaller allele being proportional to the clinical disease state [Dürr et al., 1996; Isnard et al., 1997], although the number of GAA repeats cannot fully predict the extent and course of the disease process [Klopstock et al., 1999]. The impairment of mitochondrial respiration primarily affects tissues that are the most dependent on oxidative phosphorylation to survive, the dorsal root ganglia (DRG), posterior columns, corticospinal and spinocerebellar tracts of the spinal cord, the cerebellum, cardiomyocytes and pancreatic beta cells [for review see: Marmolino, 2011; Santos et al., 2010]. Studies have found that along with general mitochondrial dysfunction, there are significant decreases specifically in activity of aconitase and complex I-III in the heart, although not in skeletal muscle, cerebellum or DRG [Bradley et al., 2000; Lodi et al., 2006].

In the present study, human FRDA fibroblasts were obtained from skin-punch biopsies of a 30-yr-old FRDA patient from Coriell Cell Repositories, a widely accepted cell model for studying FRDA [Jauslin et al., 2002, 2003, 2007; Li et al., 2008]. These cells were homozygous for the FRDA trinucleotide repeat with 541 repeats present on the first allele and 420 present on the second. BSO was used in this model to inhibit *de novo* glutathione synthesis, depleting an important component of these cell's intrinsic defenses against ROS and allowing for the accumulation of ROS produced by natural cellular

processes, resulting in cell death [Richardson et al., 2011]. The mechanism by which BSO inhibits production of GSH and results in cell death is depicted in Figure 9. Because they are lacking in frataxin, FRDA fibroblasts are extremely sensitive to BSO-induced oxidative stress compared with normal fibroblasts [Jauslin et al., 2002], and thus are used as an *in vitro* model of the long-term consequences of absent frataxin. Frataxin has been shown to be influential in the production of Fe-S cluster containing proteins [Bradley et al., 2000; Gille and Reichmann, 2011; González-Cabo et al., 2005] and to prevent intracellular ROS rise caused by the toxic effects of excess intracellular iron [Gakh et al., 2006]. In this model, the rise in ROS resulting from the lack of both frataxin and GSH impairs Fe-S cluster proteins and damages key components of the mitochondria, reducing ATP production and resulting in cell death (Fig. 9).

Here we show that phenolic ring containing estrogens are able to prevent ROS-induced damage of intracellular lipids and proteins, and are able to maintain mitochondrial function despite severe oxidative stress. All of the phenolic compounds tested have been previously shown to prevent an increase in intracellular ROS [Richardson et al., 2011], and they are further able to prevent subsequent damage caused by these ROS. Figures 4 and 5 show that phenolic estrogens prevent oxidative damage to cellular lipids and to the Krebs's cycle protein, aconitase. It has previously been established that E2 is capable of increasing levels of aconitase *in vitro* and *in vivo*, and it is thought that this effect is mediated by a reduction in ROS-related damage to aconitase [Nilsen et al., 2007; Razmara et al., 2008]. Our results indicate that ZYC-26 is able to prevent loss of aconitase activity, while ZYC-23 is not, which argues that E2 increases aconitase activity levels by reducing ROS-mediated damage to this protein in this cell

type. Phenolic estrogens also prevent damage to the mitochondria and disruption of its function, as seen by maintenance of oxidative phosphorylation and oxygen consumption rates (Fig. 6), maintenance of near normal ATP levels in cells treated with phenolic estrogens (Fig. 7), and the prevention of $\Delta\Psi_m$ collapse (Fig. 8). It has been previously shown that E2 is capable of enhancing overall mitochondrial respiration with the Seahorse assay [Yao et al., 2011]. In this study we show here that mitochondrial function is greatly impaired by BSO, and that the maximal OCR seen in BSO-treated fibroblasts is less than the resting OCR in DMSO control cells (Fig. 6a). The fact that the baseline resting OCR is statistically lower in BSO-treated fibroblasts (Fig. 6b) indicates that there is permanent damage to the mitochondria in these cells at 24 hours after BSO treatment, prior to any cell death (Fig. 3a) [Richardson et al., 2011]. This BSO-induced mitochondrial damage can be prevented by either E2 or ZYC-26, but not ZYC-23 in both resting and maximal respiratory states (Figs. 6b and 6c), indicating that the cellular respiratory depression observed in BSO-treated FRDA fibroblasts is due to oxidative damage to the mitochondria and that phenolic estrogens are acting to prevent this oxidative damage in an ER-independent manner. The Seahorse data produced in these experiments corresponds to the decrease in intracellular ATP concentration observed in BSO-treated cells (Fig. 7).

Taken together with results published previously [Richardson et al., 2011], these observations demonstrate that an oxidative insult produces a large increase in reactive oxygen species, leading to consequent lipid, protein and organelle damage, mitochondrial membrane collapse and cell death. Similar to studies in tissue samples from human FRDA patients [Bradley et al., 2000; Lodi et al., 2006], our results demonstrate that there

is significant damage to the mitochondria resulting in the inability of the ATP producing components of the cell to meet energy requirements, leading to large-scale cell death. This can be prevented by the simultaneous application of phenolic estrogens, which effectively reduces the extent of the oxidative insult of individual cells and prevents organelle damage. These data provide a potential mechanism for the protective properties of phenolic estrogens in this system.

These data support the growing body of evidence that estrogens can act to protect cells and tissues from damage inflicted by neurodegenerative disease by nongenomic means [Behl and Moosmann, 2002; Prokai and Simpkins, 2007; Rupprecht and Holsboer, 1999; Simpkins et al., 2008]. Since FRDA can be predicted and diagnosed very early [Monros et al., 1995; Pandolfo and Montermini, 1998; Wallis et al., 1989], there is a window of opportunity to begin treatment in newborns, years before any of the devastating cardiac or neurological symptoms begin to develop, a time period in which non-feminizing estrogen-like compounds may prove to be very efficacious. This study presents the first potential mechanism by which estrogens may be acting to prevent cell death in FRDA and illustrates that non-feminizing estrogens are an attractive class of candidate drugs for the prevention and delay of FRDA symptoms.

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Figure Legends

Figure 1. Structures of compounds assessed for protection against BSO toxicity in FRDA fibroblasts.

Figure 2. Western blot showing the presence of small amounts of ER β and the absence of ER α in FRDA fibroblasts compared with 661W photoreceptor cells.

Figure 3. A.) Calcein AM imaging demonstrating cell viability between vehicle control and BSO treatment groups at 24, 36 and 48 hours. Scale bar = 200 μ m. B.) Effects of E2, PPT, DPN, ZYC-26 and ZYC-23 on cell viability in BSO-treated FRDA fibroblasts. All steroid concentrations were 100nM and BSO concentration was 1mM. Depicted are mean \pm SD for n= 8 per group. * indicated p<0.05 versus BSO alone-treated cells.

Figure 4. Effects of E2, PPT, DPN, ZYC-26 and ZYC-23 on intracellular lipid peroxidation in BSO-treated FRDA fibroblasts. All steroid concentrations were 100nM and BSO concentration was 1mM. Depicted are mean \pm SD for n= 8 per group. * indicated p<0.05 versus BSO alone-treated cells. 1.0 normalized 8-isoprostane control concentration = 6.23pg/mL.

Figure 5. Effects of E2, PPT, DPN, ZYC-26 and ZYC-23 on the activity of aconitase in BSO-treated FRDA fibroblasts. All steroid concentrations were 100nM and BSO

concentration was 1mM. Depicted are mean \pm SD for n= 8 per group. * indicated $p<0.05$ versus BSO alone-treated cells.

Figure 6. Effects of E2 and ZYC-26 on mitochondrial function in BSO-treated FRDA fibroblasts. A.) Oxygen consumption rate (OCR; in pMoles/min) B.) Basal respiratory rate (in pMoles/min) C.) Maximal respiratory rate (in pMoles/min) All steroid concentrations were 100nM and BSO concentration was 1mM. Depicted are mean \pm SD for n= 8 per group. * indicated $p<0.05$ versus BSO alone-treated cells.

Figure 7. Effects of E2, PPT, DPN, ZYC-26 and ZYC-23 on the intracellular ATP content inside of BSO-treated FRDA fibroblasts. All steroid concentrations were 100nM and BSO concentration was 1mM. Depicted are mean \pm SD for n= 8 per group. * indicated $p<0.05$ versus BSO alone-treated cells. 100% normalized ATP control concentration = 501pM.

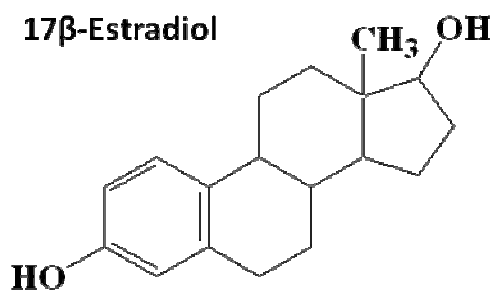
Figure 8. Effects of E2, PPT, DPN, ZYC-26 and ZYC-23 on the collapse of mitochondrial membrane in BSO-treated FRDA fibroblasts. All steroid concentrations were 100nM and BSO concentration was 1mM. Depicted are mean \pm SD for n= 8 per group. * indicated $p<0.05$ versus BSO alone-treated cells.

Figure 9. Proposed mechanism of 17 β -Estradiol in BSO-treated FRDA fibroblasts.

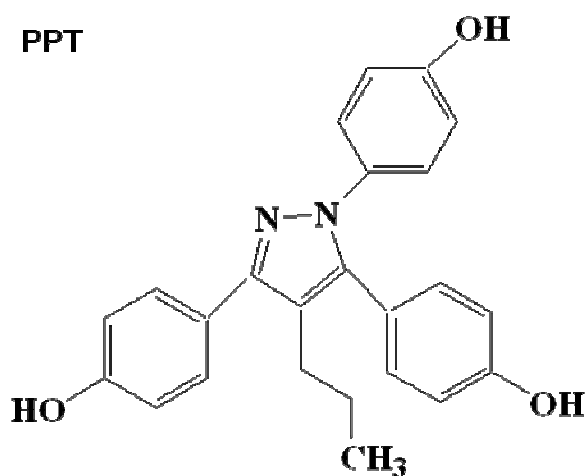
Figure 1

Estrogen Receptor Agonists

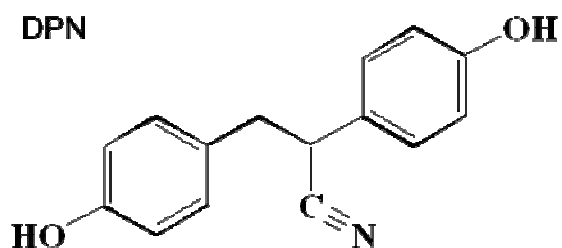
17 β -Estradiol



PPT

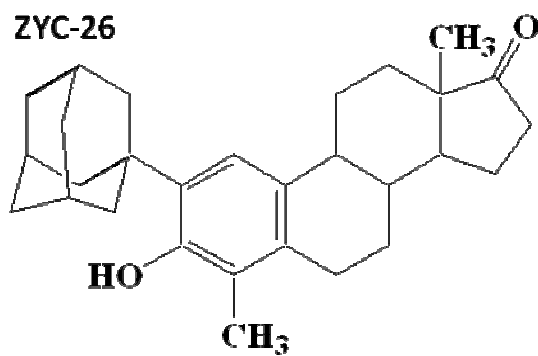


DPN



Non-ER Binding Compounds

ZYC-26



ZYC-23

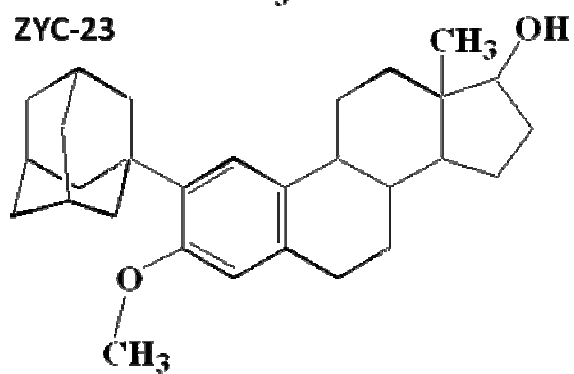


Figure 2

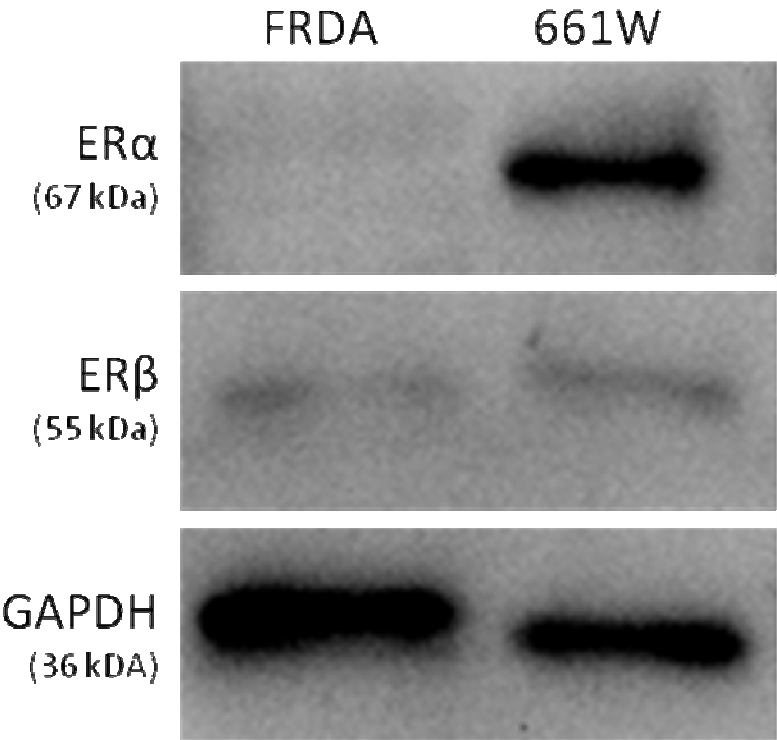


Figure 3

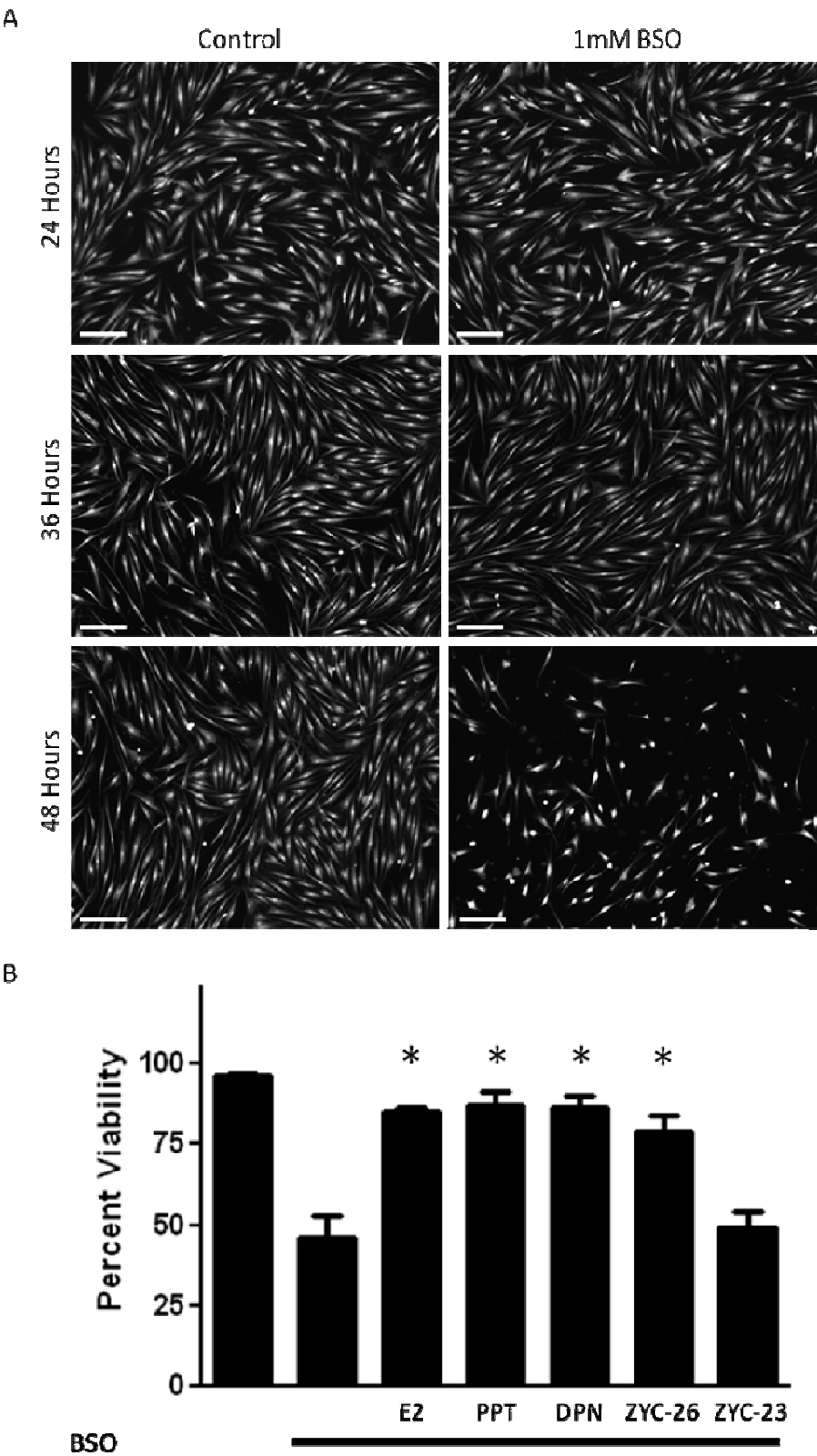


Figure 4

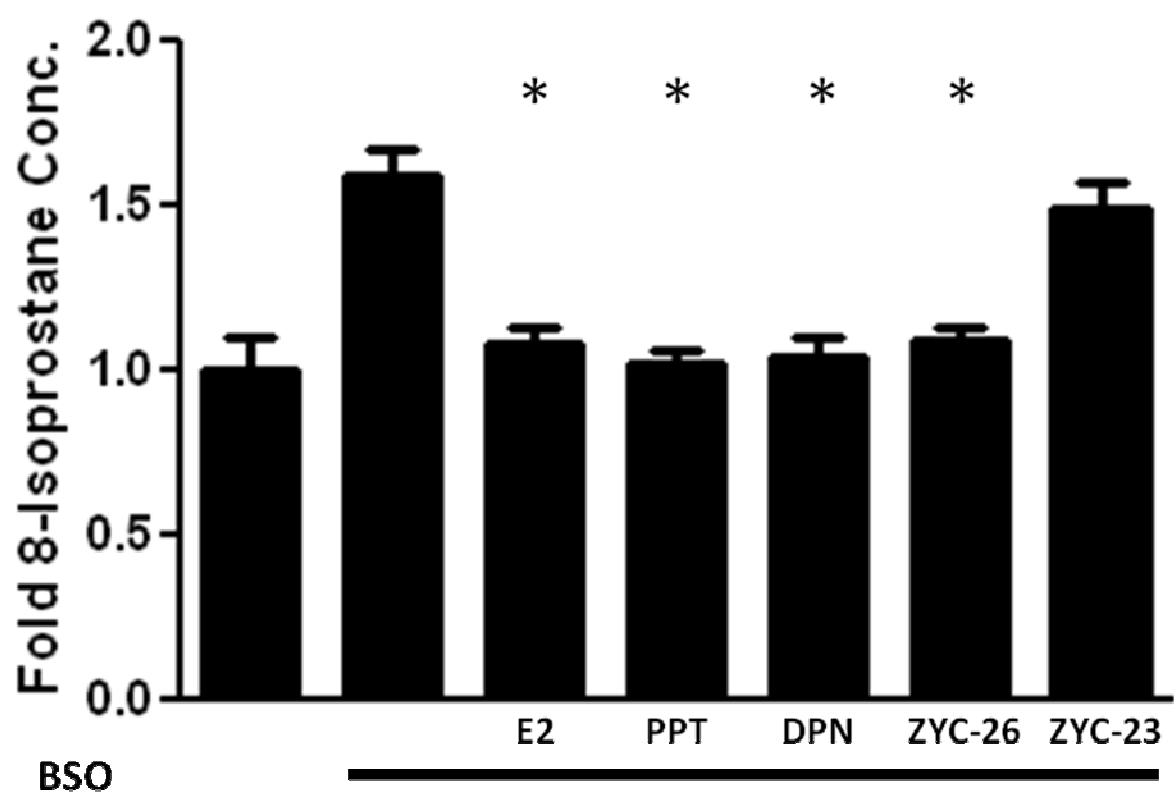


Figure 5

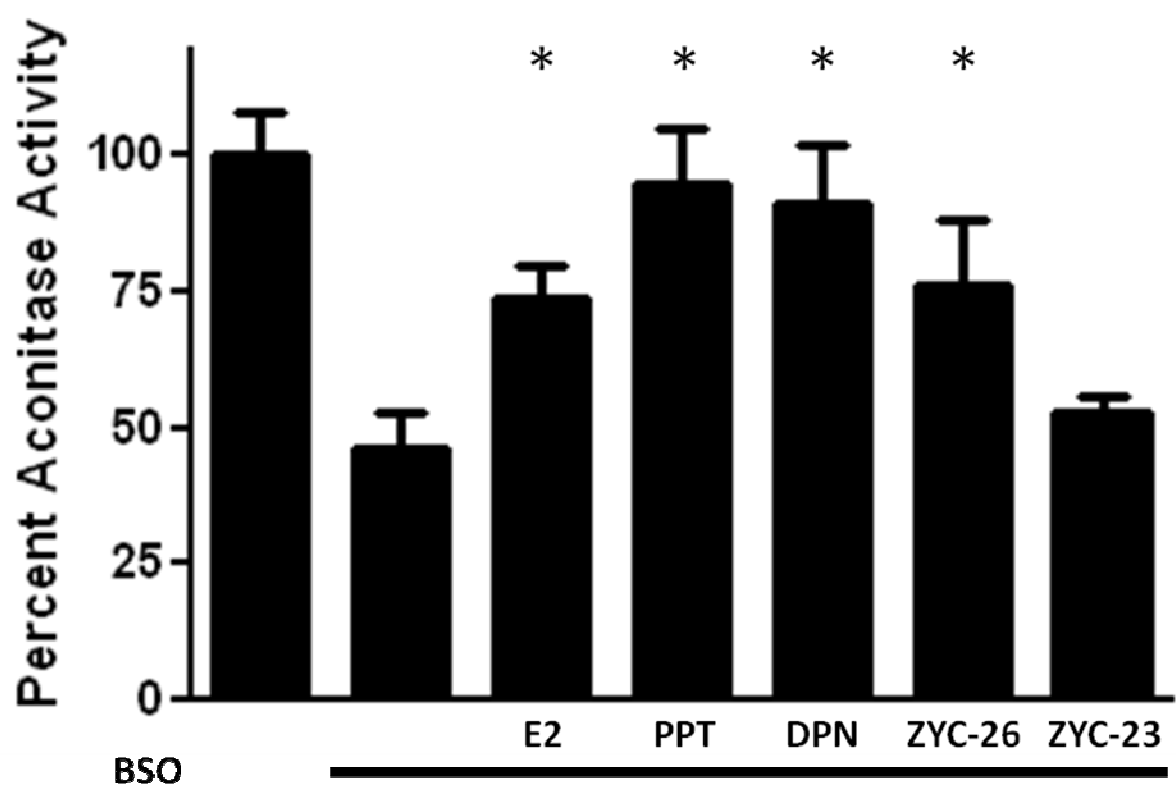


Figure 6

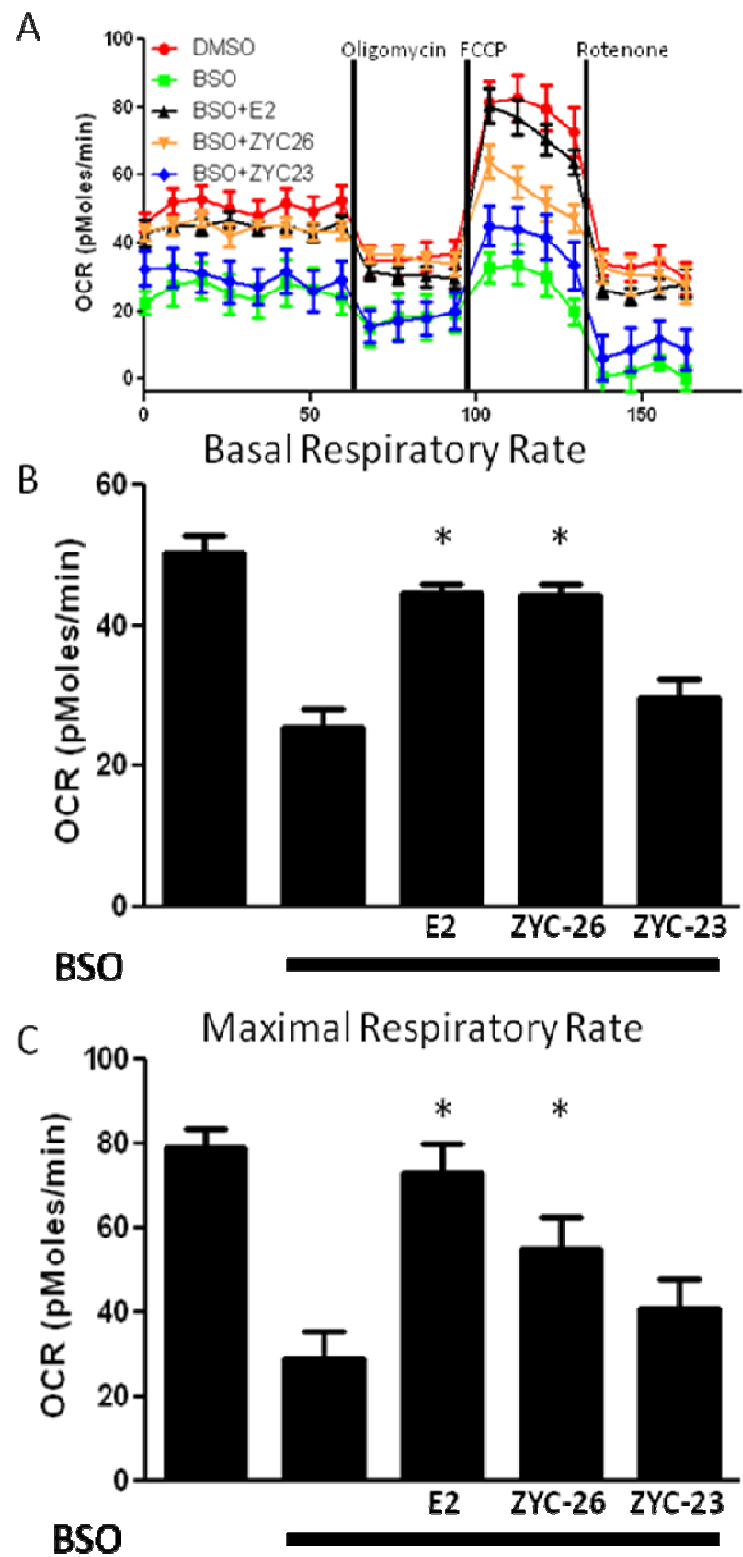


Figure 7

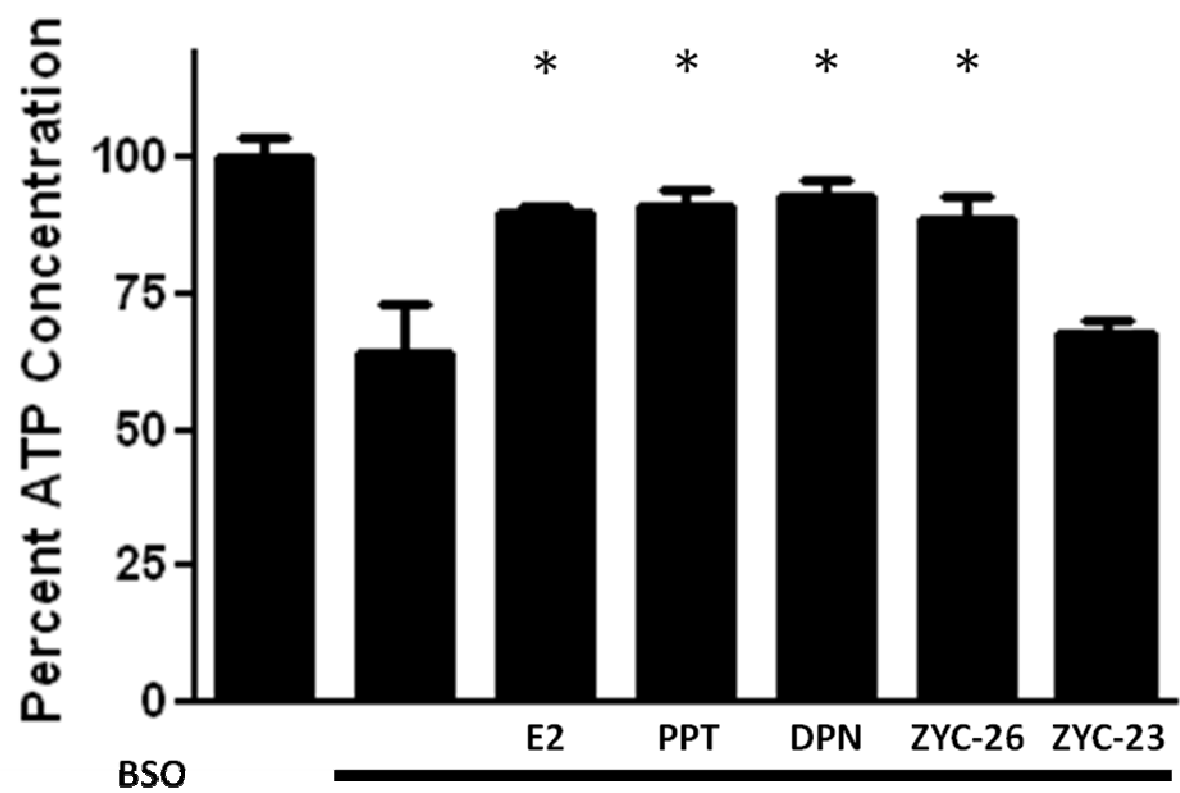


Figure 8

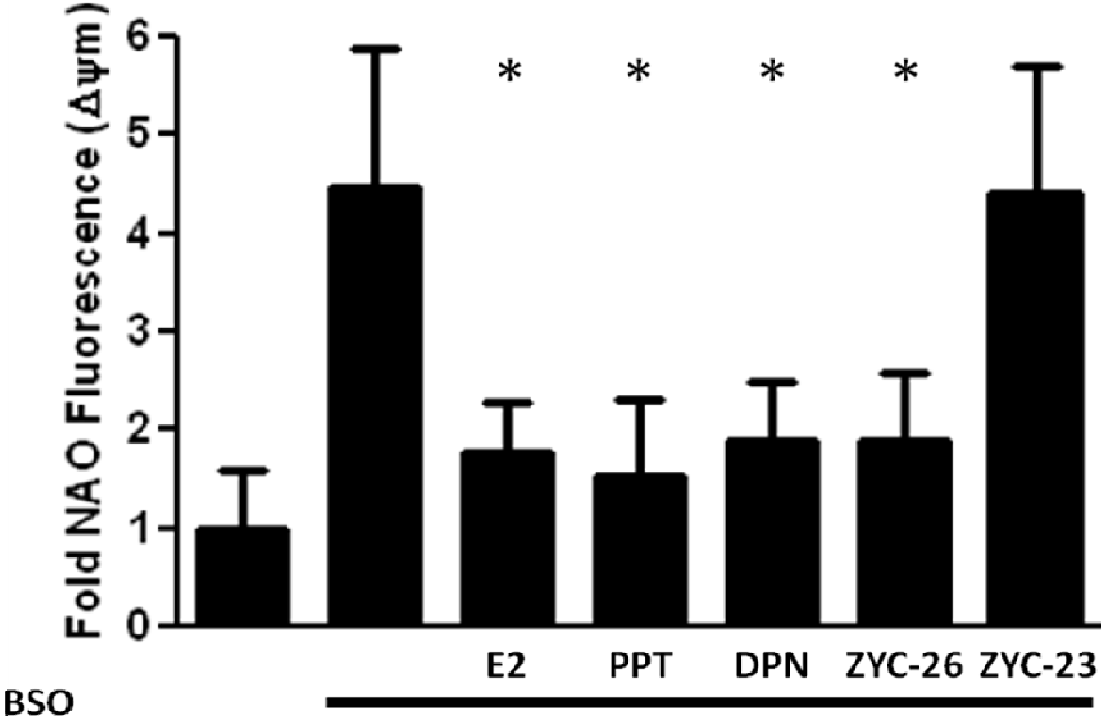
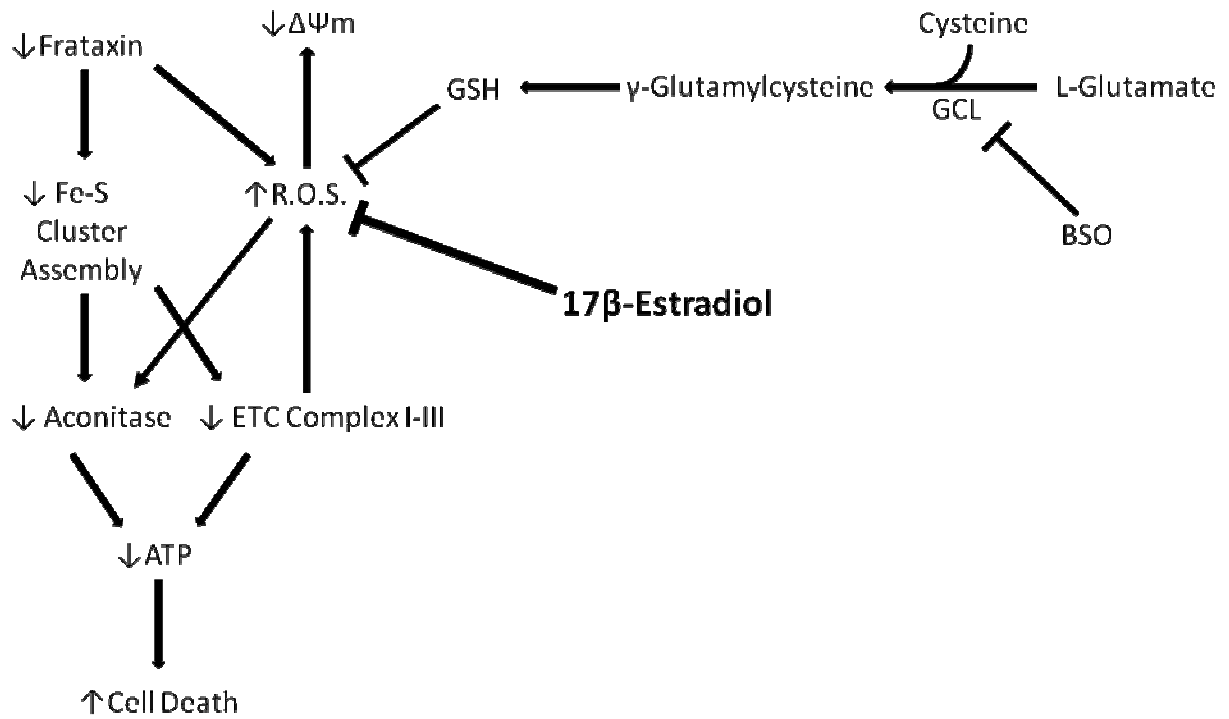


Figure 9



PREFACE TO CHAPTER V

It has been shown that both estrogens and methylene blue have significant potent cytoprotective potential against oxidative damage to the mitochondria in Friedreich's ataxia fibroblasts. Here we investigate the potential for these compounds to have additive or synergistic effects when given in combination at doses that alone would be nonprotective.

CHAPTER V

Estrogen and Methylene Blue Interactions in Friedreich's Ataxia

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Abstract

Methylene blue, estrogen and estrogen-like compounds have been demonstrated to have protective effects in many different models, including an *in vitro* model of Friedreich's ataxia. This study evaluates the possibility that these compounds could act synergistically if given in combination, alleviating many of the pharmacokinetic and pharmacodynamic problems associated with treatment, including toxic effects. Using the Friedreich's ataxia fibroblast cell model in conjunction with L-buthionine (S,R)-sulfoximine (BSO), which inhibits *de novo* glutathione (GSH) synthesis and subjects the cells to oxidative stress, we evaluate the protective potential of 1 pM-10 μ M estrogen and ZYC-26 in combination with the non-protective 1 nM methylene blue and 1 pM-10 μ M methylene blue in combination with 10 nM estrogen or ZYC-26, non-protective concentrations of these compounds. We discovered that simultaneous methylene blue treatment significantly lowers the EC_{50} value of both estrogen and ZYC-26 ~10-fold and ~12-fold respectively, and simultaneous estrogen treatment significantly lowers the EC_{50} value of methylene blue by ~11-fold, although there is no statistical change with simultaneous ZYC-26 treatment. These data show that there is a synergistic effect between these compounds, with a protective effect occurring with concentrations that would be non-protective alone. This strengthens the argument for further evaluating both estrogens and methylene blue as potential therapies for the prevention or delay of Friedreich's ataxia symptoms.

Introduction

First diagnosed by Nikolaus Friedreich in 1863 [Friedreich 1863a, 1863b, 1863c], Friedreich's ataxia (FRDA) affects 1 in 50,000 people worldwide with a carrier rate of 1 in 120 making it the most common type of inherited ataxia worldwide [Bradley et al., 2000; Campuzano et al., 1996; Harding, 1983; Leone et al., 1990; Pandolfo, 1998; Schulz et al., 2009]. The genetic basis of FRDA is a trinucleotide GAA repeat expansion in the FXN gene on chromosome 9, which normally produces frataxin protein [Fujita et al., 1989; Hanauer et al., 1990]. When this trinucleotide sequence grows beyond 100-200 repeats, a self-associating complex of triple helical DNA forms forcing histone deacetylation during DNA to mRNA transcription, effectively preventing the production of frataxin protein [Bradley et al., 2000; Campuzano et al., 1996; Grabczyk and Usdin, 2000; Heidenfelder, 2003; Lodi et al., 2006; Montermini et al., 1997; Sakamoto et al., 1999, 2001; Wells, 2008]. The precise cellular role of frataxin is still unclear, however its absence results in dysfunctional iron metabolism and impaired function of iron-sulfur (Fe-S) cluster proteins, including heme, electron transport chain (ETC) complexes and the Krebs's cycle protein aconitase, as well as dysregulation of the cellular redox state [Delatycki et al., 2000; Gakh et al., 2006; Lodi et al., 2006], ultimately leading to progressive oxidative damage to the mitochondria [Karthikeyan et al., 2003]. Similar to the pathogenesis of many other neurodegenerative diseases, this mitochondrial oxidative damage causes an impairment in aerobic ATP production and a mismatch in the ratio of ATP production to the cellular ATP demands, leading to cell death in tissues and organs most dependent on oxidative phosphorylation for survival [Bulteau et al., 2004; Chantrel-Groussard et al., 2001; Gakh et al., 2006; Jauslin et al., 2002; Santos et al., 2010].

Symptoms usually present in FRDA patients before 20 years of age with gait ataxia caused by degeneration of sensory neurons, dorsal root ganglia, spinocerebellar, corticospinal and posterior column tracts, as well as the rarer auditory and visual deficits, tremor, weakness and other sensory abnormalities, pes cavitus, lateral and kyphoscoliosis and an increased incidence of type 1 diabetes. 91% of patients have cardiac complications with hypertrophic cardiomyopathy being the most common cause of premature death [Bradley et al., 2000 ; Dürr et al., 1996; Dutka et al., 2000; Lodi et al., 2006; Geoffroy et al., 1976; Harding, 1981; Harding, 1983; Isnard et al., 1997 ; Seznec et al., 2004; Simon et al., 2004].

Currently, there is no viable treatment option for FRDA patients. Idebenone (IDB), the only drug approved for phase III trials in humans showed initial promise in both cellular [Jauslin et al., 2002, 2007] and murine models [Seznec et al., 2004] of FRDA, however it recently failed its phase III trial on the basis that it did not improve cardiac function or hypertrophic cardiomyopathy [Lagedrost et al., 2011]. Methylene blue (MB) has been used for many different indications in the past century, including for neuroprotection and improvement of neurological function in Alzheimer's disease models [Atamna and Kumar, 2010; Oz et al., 2011], retinal disease [Zhang et al., 2006], optic neuropathy [Rojas et al., 2009a], Parkinson's disease models [Rojas et al., 2009b; Wen et al., 2011], a stroke model [Wen et al., 2011] and recently in cytoprotection of FRDA fibroblasts against oxidative stress [Yu et al., 2011]. The mechanism of MB protection in neurodegenerative states is thought to be due to both its antioxidant capabilities and its ability to shuttle electrons through a damaged or otherwise nonfunctional electron transport chain [Wen et al., 2011]. Estrogen (E2) too has long

been known to have neuroprotective effects in a wide variety of neurological disorders in many different model systems [Behl, 2002; Simpkins et al., 2008], including in this cell model of FRDA [Richardson et al., 2011]. We recently hypothesized the mechanism of E2 in the FRDA cell model to be due to direct antioxidant action of the phenol ring and preservation of mitochondrial integrity independently of any known estrogen receptor [Richardson et al., 2011, 2012].

In this study, we evaluate various combinations of IDB, MB, E2 and the non-feminizing estrogen ZYC-26 [Perez et al., 2006; Richardson et al., 2011, 2012], evaluating in each a full dose-response curve ranging from 1 pM to 10 μ M with non-protective concentrations of the other compounds to establish any synergistic relationships. We show that simultaneous 1 nM MB treatment effectively increases the potency of E2 (~10-fold) and ZYC-26 (~12-fold) and treatment with 10 nM E2 increases the potency of MB (~11-fold). We observed no significant change to the potency of MB with the addition of 10 nM ZYC-26, and no interaction between E2 and IDB. Furthermore, we found no significant effects of IDB alone over the 1 pM-10 μ M range tested.

Methods

Cell Culture. Fibroblasts from a 30 year old Friedreich's Ataxia (FRDA) patient (Coriell Institute, Camden NJ, USA) were maintained in Dulbecco's Modified Eagle Medium (DMEM; ThermoScientific, Waltham, MA, USA) with 10% fetal bovine serum (FBS; ThermoScientific, Waltham, MA, USA), 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA) and 1% GlutaMAX (ThermoScientific) at 37°C in 5% CO₂ and 90% humidity. At the time of treatment, the FRDA fibroblast media was changed to phenol red- and sodium pyruvate-free DMEM (ThermoScientific) and 1% penicillin-streptomycin. All experiments were conducted using cell passages 14-19.

Chemicals & Reagents. 17 β -Estradiol (E2) was acquired from Steraloids, Inc. (Newport, RI, USA). L-buthionine (S,R)-sulfoximine (BSO) was obtained from Sigma-Aldrich (St Louis, MO, USA). Idebenone was purchased from Sigma-Aldrich. MB was obtained from Taylor Pharmaceuticals (Decatur, IL). ZYC-26 was synthesized in the laboratories of Douglas Covey [Perez et al., 2006]. Structures for all steroids are provided in Figure 1 and were drawn using ChemDraw software.

Treatment Paradigm. FRDA fibroblasts were removed from culture with 0.25% Trypsin-EDTA (Invitrogen, Carlsbad, CA, USA) and plated on 96-well plates at a density of 3,000 cells per well in DMEM with 10% CSFBS, 1% GlutaMAX and 1% penicillin-streptomycin. After 24 hours the media was removed and replaced with phenol red- and sodium pyruvate-free DMEM with 1% penicillin-streptomycin. The cells were then treated for 48 hours with either DMSO or 1mM BSO in the presence of 1 nM-10 μ M E2,

ZYC-26, MB or idebenone \pm combinations of 1-10nM E2, ZYC-26, MB or idebenone, concentrations of estrogens known to be neuroprotective in various cell lines [Jauslin et al., 2002; Richardson et al., 2011; Yi et al., 2008].

Calcein AM Cell Viability Assay. Cells were plated on a 96-well plate at a density of 3,000 cells per well, then treated with vehicle or 1mM BSO. After 48 hours of BSO treatment, the media was removed, and 1 μ g/mL Calcein AM (CalBiochem, San Diego, CA, USA) in phosphate buffer pH 7.2 (PBS; Fisher Scientific, Pittsburg, PA, USA) was added to each well and the plate was incubated for 10 minutes at 37°C. Cell viability was determined with a Tecan Infinite M200 (Tecan Systems, Inc., San Jose, CA) plate reader with an excitation of 490nm and emission of 520nm at 48 hours.

Data and Statistics. All data are displayed as mean \pm 1 standard deviation. These data were analyzed using ANOVA against an alpha level of 0.05. All bar graphs were made using GraphPad Prism 5 and EC₅₀ calculations were made with GraphPad Prism 5. For all groups, n=8 wells and experiments were repeated three times to ensure consistency.

Results

Effects of estrogen and methylene blue on cell viability in BSO-treated FRDA

fibroblasts. A dose response curve of MB was evaluated from 1 pM to 10 μ M with and without the non-protective E2 dose of 10 nM (Figs. 2b and 4b) in BSO-treated cells. We found significant protection in the BSO and MB-alone treated cells between 10 nM and 1 μ M, with maximal effects at 100 nM and toxicity at 10 μ M. With the addition of 10 nM E2 the curve shifted significantly to the left indicating an increase in potency of MB.

There was also a significant protective effect at 1 nM MB in the presence of estrogen, an increase in efficacy from 10 nM-1 μ M and protection from toxicity at 10 μ M (Fig. 2a).

The addition of estrogen significantly reduced the EC₅₀ of MB from 9.7 nM to 880 pM (Fig. 5 and Table 1). A dose response curve of E2 was also evaluated from 1 pM to 10 μ M with and without the non-protective MB dose of 1 nM (Fig. 2a) in BSO-treated cells. There was again a significant left-shift of the E2 viability curve with 10 nM-10 μ M being protective in the presence of MB, while only 100 nM-10 μ M was protective without it (Fig. 2b). There was also a small increase in efficacy between 100 nM-10 μ M with 1 nM MB and an ~11-fold decrease in the EC₅₀ (Fig. 5 and Table 1).

Effects of ZYC-26 and methylene blue on cell viability in BSO-treated FRDA

fibroblasts. In this study, a dose response curve of cell viability in BSO-treated cells was run with 1 pM to 10 μ M of MB with and without the non-protective 10 nM (Fig. 3b) of the non-feminizing estrogen compound ZYC-26 in BSO-treated cells. Here we found no significant increase in efficacy or potency (Fig. 5 and Table 1), although there was a small but significant protective effect against the toxic effects of 10 μ M MB in the ZYC-

26 treated cells (Fig. 3a). Conversely, figure 3b shows that in FRDA fibroblasts treated with 1mM BSO and 1 pM-10 μ M ZYC-26 there is no significant increase in efficacy produced by the addition of 1 nM MB to any of the ZYC-26 doses, however there is a significant increase in potency as illustrated by a left shift of the dose-response curve and ~12-fold increase in ZYC-26 potency with EC₅₀ values falling from 55.41 nM and 4.64nM with the addition of 1 nM MB present.

Effects of E2 and idebenone on cell viability in BSO-treated FRDA fibroblasts. We ran a dose response curve of idebenone from 1 pM-10 μ M with and without the non-protective E2 dose of 10 nM (Figs. 2b and 4b), and did not find any significant protection of idebenone by itself with significant toxicity at 10 μ M. The addition of 10 nM estrogen to this curve did not detectably increase the potency, although there appears to be a small increase in efficacy in the 1-10 nM idebenone range (Fig. 4a). No EC₅₀ value could be determined for idebenone with or without 10 nM E2. In the dose response curve of E2 from 1 pM to 10 μ M \pm the non-protective idebenone dose of 10 nM (Fig. 4a) there was also no detectable significant difference to the potency or efficacy of E2 in the presence of idebenone. There was, however, a consistent trend toward the E2 curve with 10 nM idebenone being more efficacious at each dose of E2 and a general trend toward a left shift of the E2 dose response curve. There was no statistically significant change in the EC₅₀ of E2 in the presence of idebenone (Fig. 5 and Table 1). The lack of idebenone efficacy was a surprising result as other groups have found that there was a protective effect of idebenone in other FRDA fibroblast cell lines [Jauslin et al., 2003, 2007]. The specific cell line of FRDA fibroblasts used in these experiments has been previously

shown not to respond favorably to idebenone treatment [Unpublished Observations, Yu and Yang, 2011].

Discussion

FRDA fibroblasts, including the cell line used here, have been utilized as an initial screening tool for drugs designed to treat FRDA by several different groups [Jauslin et al., 2002, 2003, 2007; Richardson et al., 2011, 2012; Yu et al., 2011]. Taken from skin-punch biopsies used to diagnose FRDA clinically, the fibroblasts used here were taken from a 30-year old male Caucasian FRDA patient with clinical signs of the disease and homozygous GAA expansion, with 541 trinucleotide repeats on the first allele and 420 on the second. These cells also contain no detectable ER α protein and only small quantities of ER β as measured by western blot [Richardson et al., 2012]. Friedreich's ataxia can be understood as a genetic disorder as well as a metabolic disorder [Santos et al., 2010]. The lack of frataxin caused by GAA repeats in the first intron of the FXN gene in this cell type impairs the function of Fe-S cluster proteins, which is complicated by oxidative stress, produced in high quantities in these cells by the failing mitochondria and increased free iron molecules via Fenton chemistry further inhibiting oxidative phosphorylation complexes and aconitase [Bulteau et al., 2004; Chantrel-Groussard et al., 2001; Delatycki et al., 2000; Lodi et al., 2006; Santos et al., 2010]. BSO is to inhibit the rate limiting step of glutathione synthesis, artificially raising the levels of ROS produced naturally in cells and organ systems afflicted by FRDA [Jauslin et al., 2002, 2003; Richardson et al., 2011, 2012] to mimic the redox conditions found in human FRDA. FRDA fibroblasts are significantly more vulnerable to the oxidative stress induced by BSO than regular fibroblasts [Jauslin et al., 2002; Yu et al., 2011], and so are used here as an *in vitro* model of FRDA.

Phenol rings on E2 and other estrogens including the ER α agonist PPT, the ER β agonist DPN and other non-feminizing estrogens give these compounds very potent antioxidant properties [Richardson et al., 2011]. Estrogens are ideally designed to penetrate the blood brain barrier and insert into membranes stopping the cascade of ROS, lipid and protein oxidation and mitochondrial damage by a cyclic and reusable phenol-quinol reaction [Prokai et al., 2003, 2006; Prokai and Simpkins, 2007]. In the FRDA fibroblast cell line, we have shown that there is a potent cytoprotective effect with any phenol ring containing estrogen-like compound, including both E2 and the non-feminizing ZYC-26, attenuating ROS, preventing lipid peroxidation and Fe-S enzyme damage, maintaining mitochondrial function and intracellular ATP concentration and preventing mitochondrial membrane potential ($\Delta\Psi_m$) collapse and cell death [Richardson et al., 2012]. All of these effects are independent of any known estrogen receptor, and are believed to be dependent on the direct antioxidant properties of phenol rings with the number of phenol rings present in each compound directly correlated to the potency of that compound [Richardson et al., 2011]. MB is also able to attenuate the ROS and cell death induced by BSO in this cell type, as well as preventing the reduction in complex I-III and IV activity and $\Delta\Psi_m$ collapse in response to BSO treatment [Yu et al., 2011]. The mechanism of MB has been hypothesized to act as both an antioxidant and electron shuttle in oxidative phosphorylation, supporting the mitochondria against a variety of *in vitro* and *in vivo* insults [Wen et al., 2011].

In the present study, we assessed for any possible additive or synergistic reactions between E2, ZYC-26, MB and idebenone in terms of an increase in either potency or efficacy. We found that the addition of 1 nM MB increases both the potency of E2 and

ZYC-26, ~10-fold and ~12-fold respectively (Fig. 5 and Table 1). In addition, the non-protective 1 nM concentration of MB (Fig. 2a) significantly increases the efficacy of E2 in the 100 nM to 10 μ M range and causes E2 to have a significant protective effect at 10 nM while there is no protective effect at this concentration in the BSO- and E2-alone treated cells (Fig. 2b). The addition of the non-protective concentration of 10 nM E2 also produces an increase in MB potency of ~11 fold (Fig. 5 and Table 1). There is also a significant increase in efficacy between 10nM-1 μ M, a new protective effect at 1 nM and a small but significant protection from the toxic effects of MB at 10 μ M (Fig. 2a). We found no increase in the potency or efficacy of MB with the addition of the non-protective 10 nM ZYC-26 (Fig. 5 and Table 1). There was, however, still a protective effect against the toxic effects of 10 μ M MB with 10 nM ZYC-26 (Fig. 3a). Idebenone, which has been shown to be protective in FRDA fibroblasts [Jauslin et al., 2002, 2007], although not in this specific line of FRDA fibroblasts [Unpublished Observations, Yu and Yang, 2011], did not have any discernable protective effects in the 1 pM-10 μ M range tested, although it did have a very large toxic effect at 10 μ M (Fig. 4a). We found no significant interactions between E2 and idebenone, but there were non-significant trends toward additive effects. Figure 4a shows that 10nM E2 increases the efficacy of idebenone in the 1 nM-100 nM range, but offers no significant protection against the toxicity of 10 μ M idebenone. There was also a consistent trend toward an increased protective effect of E2 from 1 pM to 10 μ M with the addition of 10nM idebenone, although there was no measurable increase in potency (Fig. 5 and Table 1).

In conclusion, these data show that both E2 and MB are viable candidate drugs for the treatment and prevention of FRDA signs and symptoms, either alone or in

combination. With the recent failure of idebenone in phase III trials [Lagedrost et al., 2011], new compounds are needed to prevent the devastating neurological and cardiac damage seen in FRDA patients. Because of their respective mechanisms of action, E2 and MB are both ideal candidates for correcting the intracellular redox state and maintaining mitochondrial integrity, however at doses high enough to produce effects in both cardiac and neurological tissues their toxic effects may outweigh any protective effects. This study is the first to show that in combination, these two drugs produce cytoprotective effects at much lower concentrations, allowing for the possibility of significant cardio- and neuroprotection at much lower levels of each, well below their toxic range.

Acknowledgements: We would like to thank Maninder Malik for all of her help with ChemDraw software in figure 1.

Figure Legends

Figure 1. Structures of compounds assessed for protection against BSO toxicity in FRDA fibroblasts.

Figure 2. A.) Effects of MB in the presence (■) and absence (○) of 10 nM E2. BSO concentration is 1 mM and MB concentration varies from 1 pM to 10 μM. * indicated $p < 0.05$ versus MB alone-treated cells. B.) Effects of E2 in the presence (■) and absence (○) of 1 nM MB. BSO concentration is 1mM and E2 concentration varies from 1 pM to 10 μM. * indicated $p < 0.05$ versus E2 alone-treated cells.

Figure 3. A.) Effects of MB in the presence (■) and absence (○) of 10 nM ZYC-26. BSO concentration is 1 mM and MB concentration varies from 1 pM to 10 μM. * indicated $p < 0.05$ versus MB alone-treated cells. B.) Effects of ZYC-26 in the presence (■) and absence (○) of 1 nM MB. BSO concentration is 1mM and ZYC-26 concentration varies from 1 pM to 10 μM. * indicated $p < 0.05$ versus ZYC-26 alone-treated cells.

Figure 4. A.) Effects of idebenone in the presence (■) and absence (○) of 10 nM E2. BSO concentration is 1 mM and idebenone concentration varies from 1 pM to 10 μM. * indicated $p < 0.05$ versus idebenone alone-treated cells. B.) Effects of E2 in the presence (■) and absence (○) of 10 nM idebenone. BSO concentration is 1mM and E2 concentration varies from 1 pM to 10 μM. * indicated $p < 0.05$ versus E2 alone-treated cells.

Figure 5. Comparison of EC₅₀ values for all of the interactions measured: E2, E2 + 1 nM MB, E2 + 10 nM idebenone, ZYC-26, ZYC-26 + 10 nM MB, MB, MB + 10 nM E2 and MB + 10 nM ZYC-26. * indicated p<0.05 versus E2, ZYC-26 or MB alone-treated cells.

Figure 1.

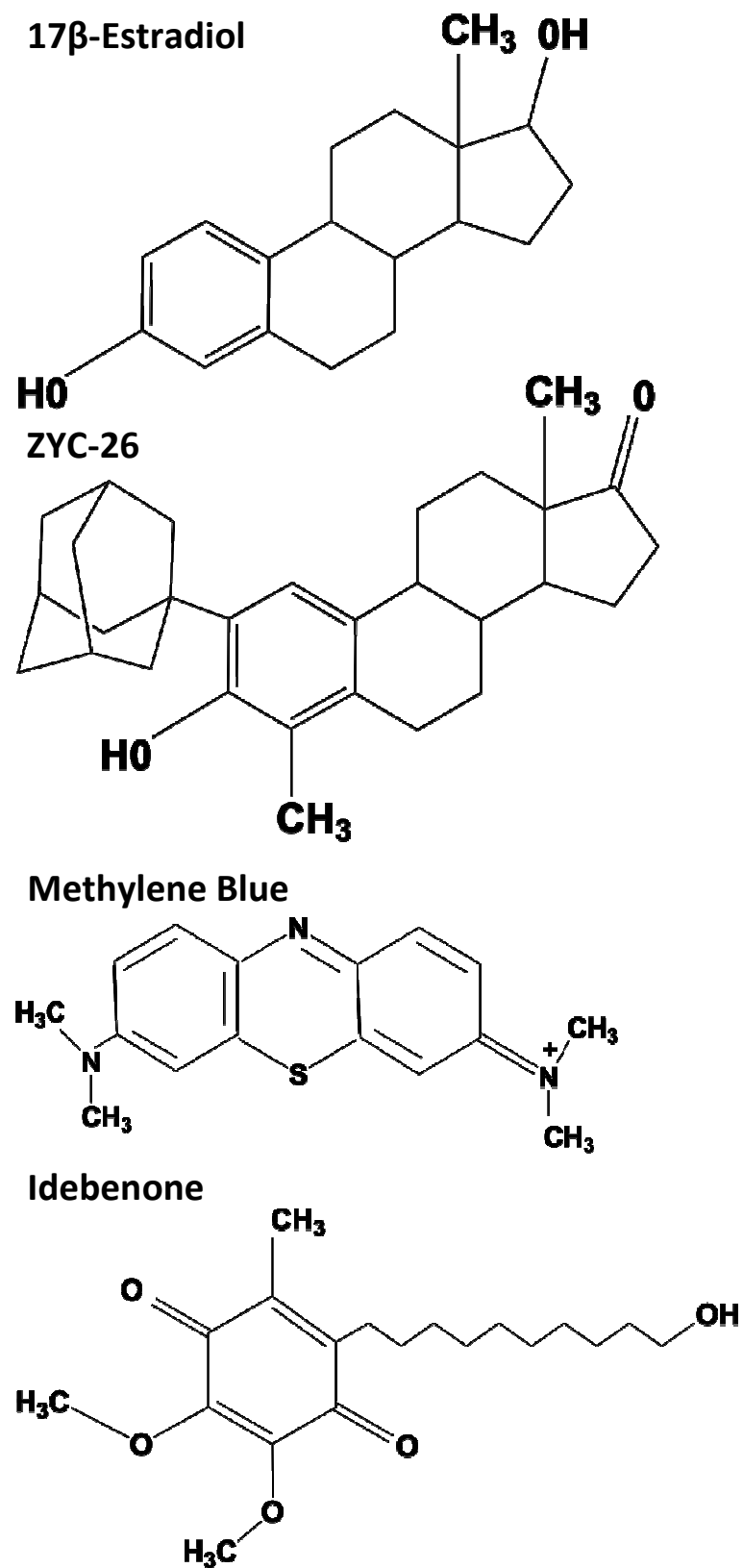


Figure 2.

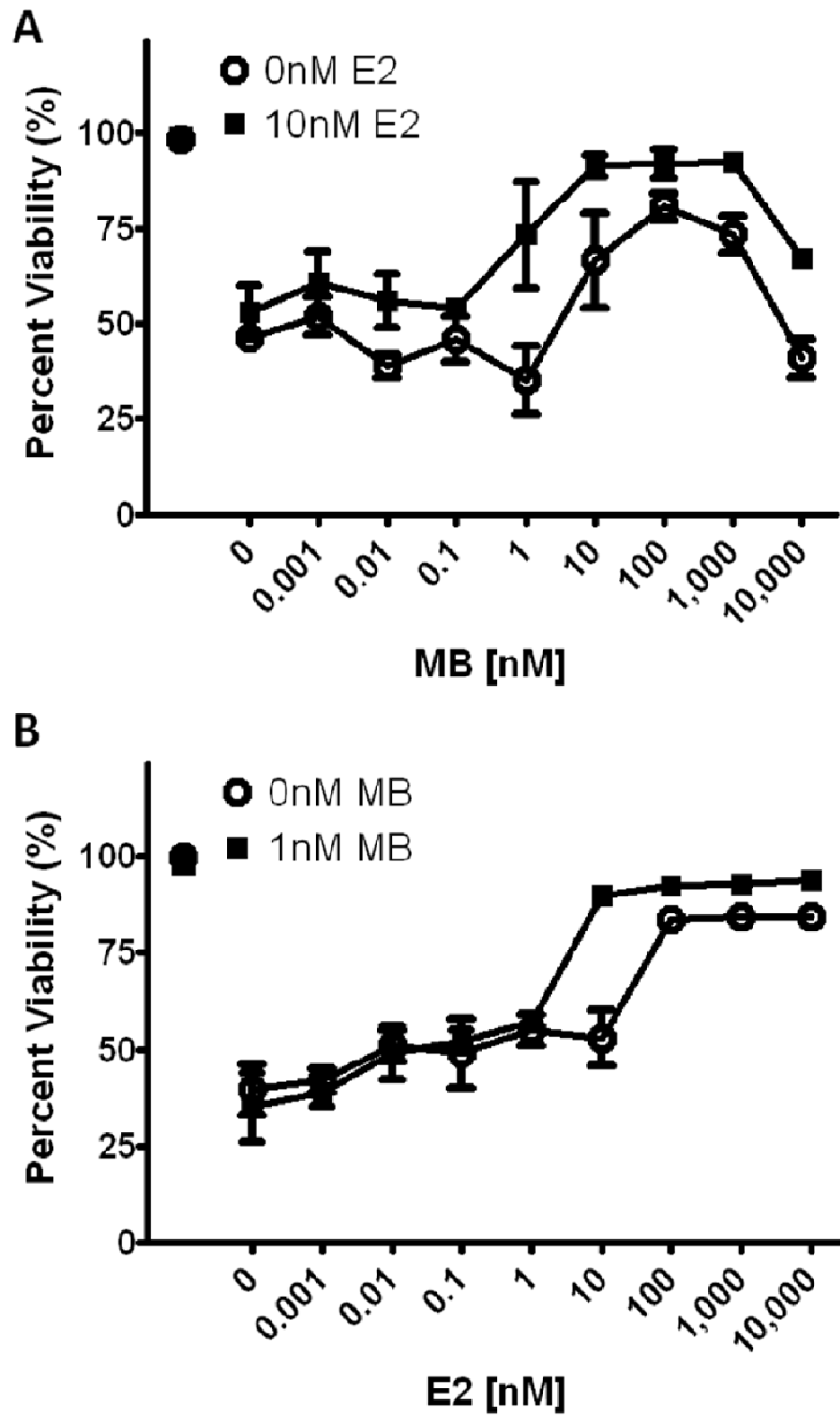


Figure 3.

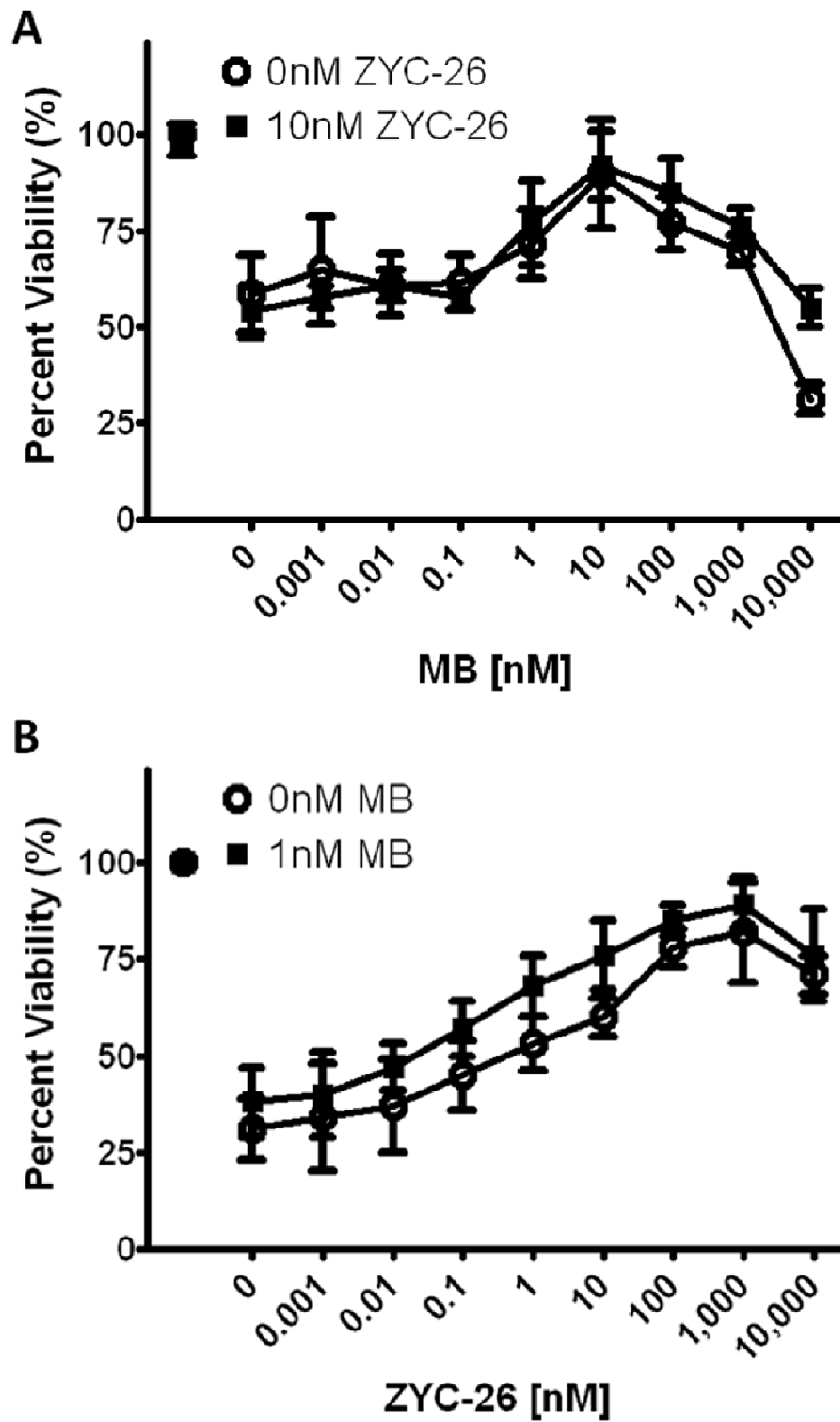


Figure 4.

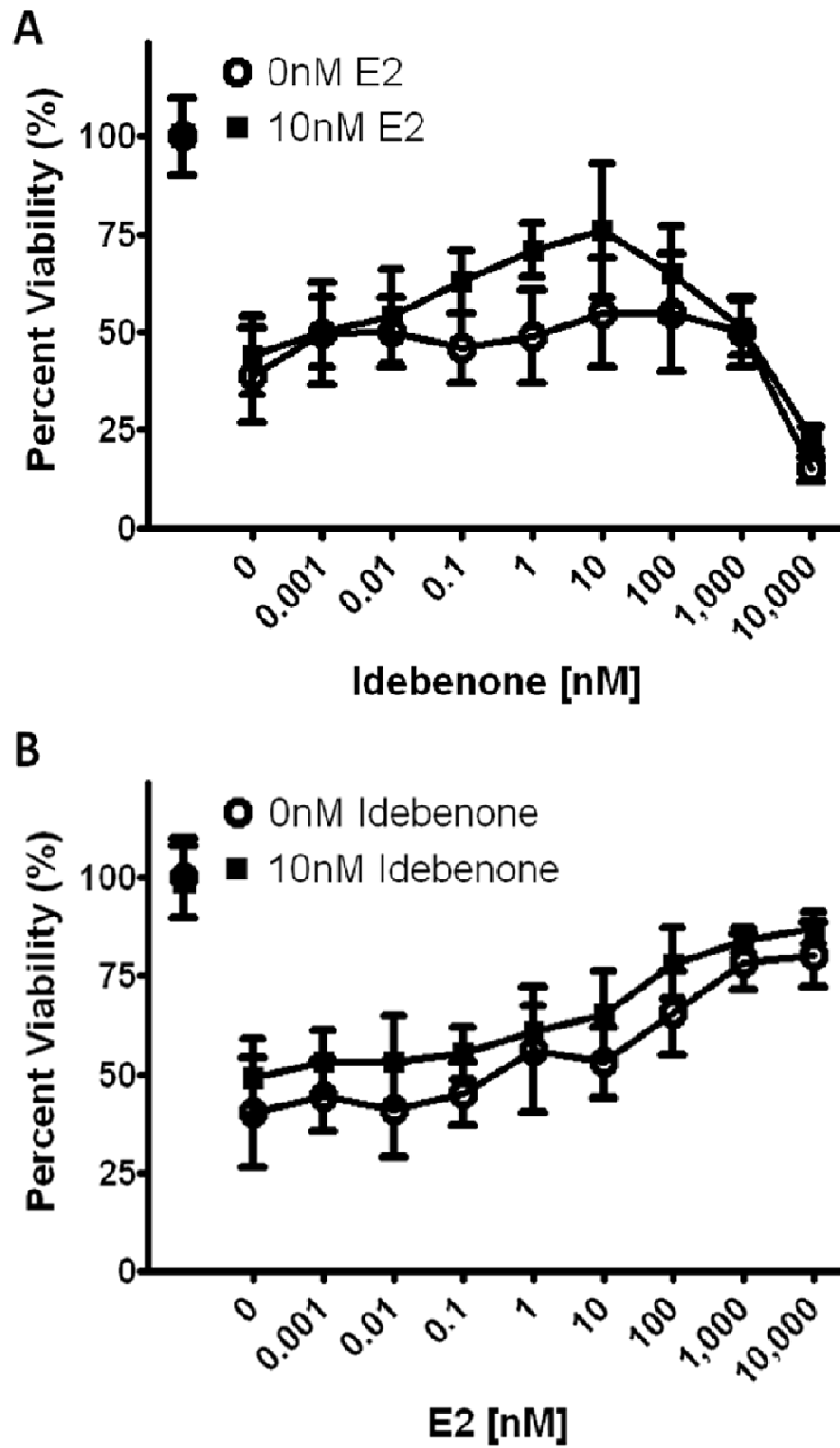


Figure 5.

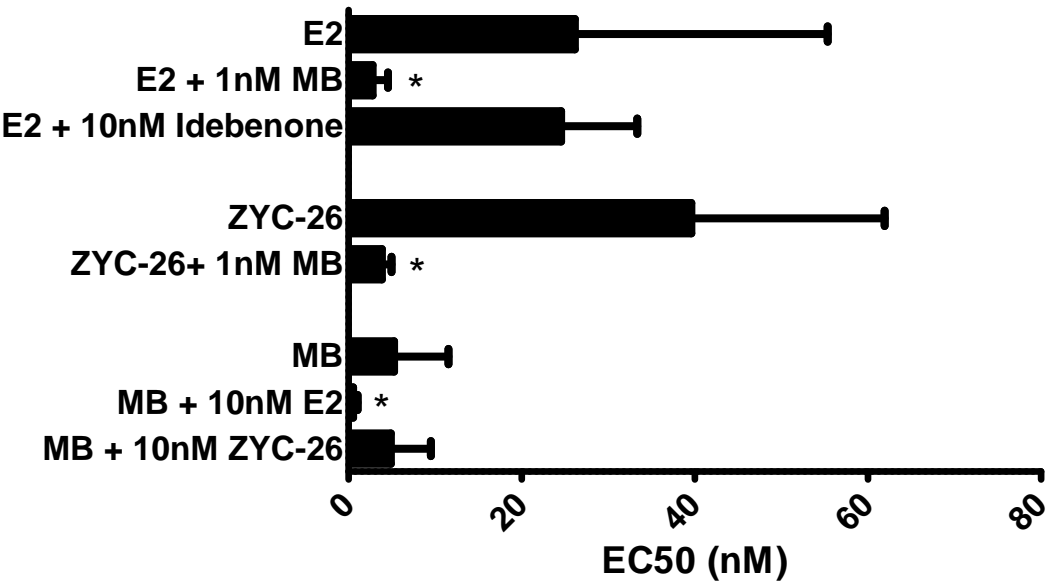


Table 1. EC₅₀ values for interaction studies.

Cell Viability EC₅₀

Compound	EC₅₀ (nM)	95% Confidence Interval (nM)
E2	46.79	39.63 - 52.27
E2 + 1nM MB	4.03	2.46 - 5.55
E2 + 10nM Idebenone	30.77	17.71 - 49.12
ZYC-26	55.41	26.61 - 79.21
ZYC-26 + 1nM MB	4.64	1.03 - 8.28
MB	9.70	9.31 - 10.09
MB + 10nM E2	0.88	0.76 - 1.03
MB + 10nM ZYC-26	8.14	7.45 - 9.73

CHAPTER VI

Conclusions & Future Directions

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Friedreich's ataxia (FRDA) can be viewed as both a genetic disease and a mitochondrial disease. While the GAA trinucleotide repeat in the first intron of the FXN gene gives it a unique cause among neurodegenerative diseases, the oxidative damage to and eventual collapse of mitochondrial function, resulting from the lack of the FXN gene product frataxin, is a common link between it and many other age-related neurological disorders such as Alzheimer's disease, Parkinson's disease, Huntington's disease and ischemic stroke [Beal, 2000; Gibson et al., 1998; Lenaz et al., 2006, 2010; Mizuno et al., 1989; Parker et al., 1989; Simpkins and Dykens, 2008]. Estrogens have been shown to be very potent and effective neuroprotectants in many *in vitro* and *in vivo* models of these diseases [Behl, 2002; Simpkins et al., 2008, 2009, 2010], although there has been considerable difficulty in translating this to results in human studies, most notably the Women's Health Initiative (WHI) study [Simpkins and Singh, 2008]. Many of the problems associated with using estrogen or other hormone therapies in humans comes from the problems associated with ER α binding, which has led to the development of selective estrogen receptor modulators (SERMs) and other non-feminizing estrogens, which are inactive at either ER α or both ER α and ER β [Perez et al., 2006]. Our studies have focused on certain properties of 17 β -estradiol, most notably the presence of a phenol in the A ring of the molecule [Prokai et al., 2005, 2006; Prokai and Simpkins, 2007; Richardson et al., 2011; Richardson et al., 2012], and include a wide range of estrogen-like molecules with varying numbers of phenol rings and estrogen receptor binding capabilities (Table 1) to evaluate a possible mechanism for estrogens to act in an ER-independent manner. Our cell type, human FRDA fibroblasts, provide an ideal environment to study the effects of ER-independent action. Despite previous reports

indicating that certain human fibroblasts contain ER α , ER β and the putative membrane ER GPR30 [Haczynski et al., 2002; Madeo and Maggiolini, 2010], our own studies suggest that these FRDA fibroblasts do not contain ER α and only very small amounts of ER β [Richardson et al., 2012] which doesn't seem to contribute to the potency or efficacy of estrogen-like compounds in this model [Richardson and Simpkins, 2012].

We began these experiments by testing the percent viability of FRDA fibroblasts under a series of conditions [Richardson et al., 2011]. L-buthionine (S,R)-sulfoximine (BSO) was used in these experiments to inhibit the *de novo* synthesis of glutathione (GSH) [Jauslin et al., 2002, 2003], impairing the cell's defenses against oxidative stress, resulting in cell death 48 hours after treatment. We showed that any compound tested with a phenol ring (E2, DPN, PPT, ZYC-26, R- and S-Equol) very potently prevented this widespread cell death, while those without phenol rings (ZYC-23, G1 and G15) did not. This effect was ER-independent, as it was not blocked by any ER antagonist used, and in fact the ER inhibitor ICI 182,780, which includes a phenol ring as part of its structure, was protective at concentrations of ~ 2 μ M. In addition, the EC₅₀ values were measured for each of the phenolic steroids (Table 1), and it was determined that there is an inverse relationship between the potency of the compound in terms of EC₅₀ and the number of phenol rings present in the structure. Figure 1 shows that compounds with one phenol ring (E2 and ZYC-26) have EC₅₀ values ranging from 15.5-23.1 nM, while DPN with 2 phenol rings has an EC₅₀ of 9.6 nM and PPT with 3 phenol rings has an EC₅₀ of 4.6 nM. Compounds with no phenol ring (G1, G15 and ZYC-23) had no measurable protective properties and were given an EC₅₀ of $>10,000$ nM, the highest concentration measured [Richardson et al., 2011].

We further investigated the protective phenomenon in this cell model by attempting to determine the mechanism of E2 protection. We assayed for reactive oxygen species with a DCFDA assay [Richardson et al., 2011], lipid peroxidation, aconitase activity, ATP production, oxidative mitochondrial function, GSH concentration and mitochondrial membrane potential collapse [Richardson et al., 2012], as well as frataxin protein levels (see appendix II). It was determined that phenol ring-containing estrogens attenuated ROS, thus preventing lipid peroxidation and damage to mitochondrial proteins such as aconitase. This preserved ATP production and oxidative mitochondrial function in terms of oxygen consumption rate and prevented the collapse of mitochondrial membrane potential. The decreased levels of GSH in the BSO treated cells was not altered, nor was the levels of frataxin increased in estrogen treated cells. From these data, we concluded that in this system, estrogens are acting as antioxidants, preventing the oxidative damage to the cells that is seen in BSO treated cells and with individuals with FRDA [Edmond et al., 2000; Lodi et al., 2006; Pandolfo, 2002; Schulz et al., 2000].

These data represent a start to determining the true potential for estrogens, specifically non-feminizing estrogens to be used as a treatment for FRDA patients. While these experiments have shown that estrogens can protect very basic cell types in an *in vitro* environment, much work remains to be done to determine their efficacy as clinical drugs for this condition. To this end, *in vitro* studies must be translated to *in vivo* ones before the chosen compounds can be assessed for clinical trials. We know these drugs are very potent cytoprotectants, however in whole animal studies a wide range of other factors must be considered. Idebenone, the prototype antioxidant and mitochondrial stabilizer in FRDA, has not proven to be a very effective drug clinically [Santos et al.,

2010]. While it has been shown to improve cardiac function and pathology, most studies have not found any improvement in the ataxia or other neurological components of the disease, and while the mortality-related effects are statistically significant, they represent only a meager increase in life. These findings illustrate the myriad of other factors that must be taken into account; pharmacokinetic and pharmacodynamic factors are important considerations *in vivo*, as is the effect on cells that do contain ER α or ER β , and a viable drug candidate must be active past the blood brain barrier (BBB). Timing of drug delivery is also vital to the success of a potential drug. Since the FRDA genotype is present at birth, but the phenotype does not reveal itself until childhood to puberty, there is a significant window of time to begin treatment to prevent the mitochondrial damage that is a hallmark of this condition before symptoms begin. This window is important to understand, as many investigators believe that the past failure of estrogen-like drugs in other disease states may be due to an incorrect understanding of the degenerative process, resulting in administration far too late in the course of the disease, a time point in which hormone therapies may well do more harm than good. Clinical trials must utilize this window if they are to be successful, since the destruction of the specific spinal cord columns and other neurological, cardiac and endocrine tissues is generally irreversible. Estrogens are potentially a very powerful tool in ameliorating the devastating disease process in FRDA, however before they can be introduced as candidate drugs many more studies must be conducted. There are several different murine models of FRDA, and estrogens would first have to successfully be shown to prevent or delay symptom onset in these animals. This requires both behavioral and pathologic data showing improvement in disease progression. Drugs would also have to be screened in phase I and phase II trials.

Several ER β agonists are already in clinical trials for other indications, such as LY500307 [<http://clinicaltrials.gov/ct2/show/NCT01097707>]. We have shown that this compound is somewhat protective in our human FRDA fibroblast cell line (see appendix III), indicating that it is a more likely candidate for use in future animal and human studies.

Overall, we have made the first step in establishing estrogen-like compounds as a possible therapy for FRDA. While there is much work to be done, these data are encouraging in that they represent another avenue to pursue in developing therapeutics for this condition, and provide another framework for understanding the sequence of intracellular events that lead from the genetic abnormality to the visible signs and symptoms in patients. Lastly, estrogens have been widely shown to have beneficial effects *in vivo* and *in vitro* for other degenerative disease [Behl, 2002], so a success in treating FRDA with these drugs could potentially be used to understand and develop treatment strategies for other neurodegenerative diseases with similar mitochondrial mechanisms of action.

Figure Legends

Figure 1. Graph showing the inverse relationship between the number of phenol rings in the molecular structure vs the ED₅₀ of the compounds (in nM). E2, PPT, DPN, ZYC-26 and G1 are used in this figure.

Figure 1

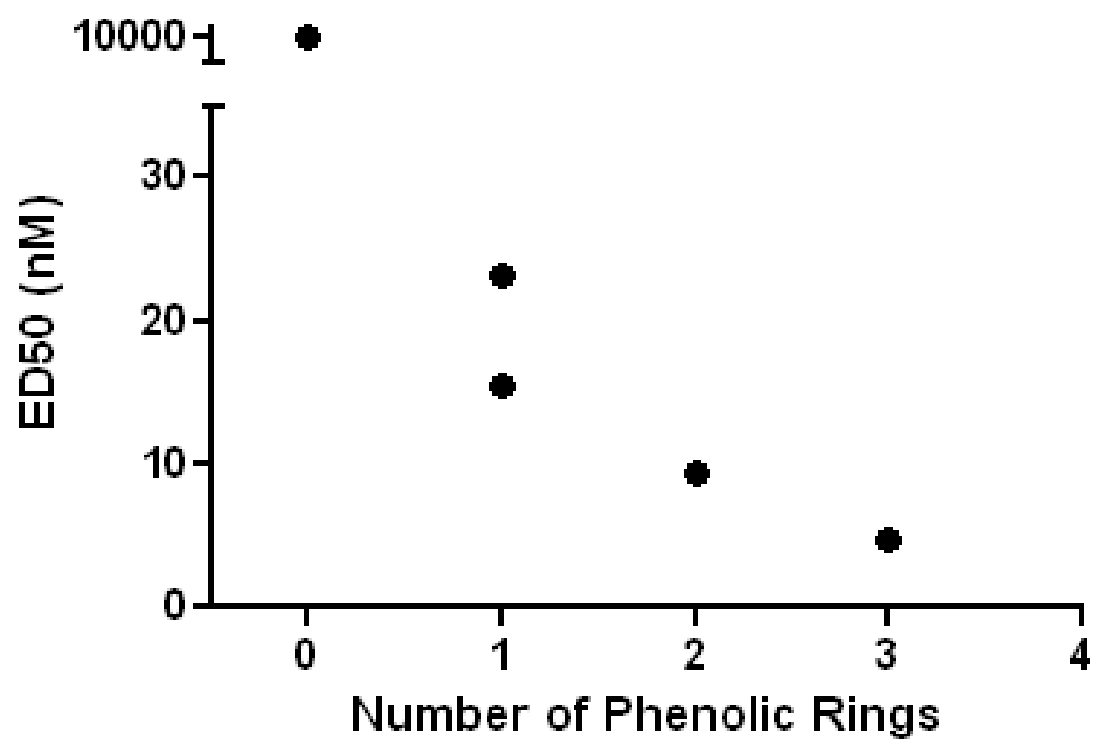


Table 1. Steroids used and compound properties.

Compound Name	ER Binding	Phenol Rings	ED ₅₀ (nM)	95% Confidence Interval
17 β -Estradiol (E2)	ER α /ER β /GPR30	1	15.5	10.2-23.5
PPT	ER α	3	4.6	1.7-12.3
DPN	ER β	2	9.3	7.2-12.1
G1	GPR30	0	>10,000	
G15	GPR30 Antagonist	0	>10,000	
ZYC-23	N/A	0	>10,000	
ZYC-26	N/A	1	23.1	11.9-44.9
ICI 182,780	ER α /ER β Antagonist	1	>500	

APPENDIX I

Friedreich's Ataxia Fibroblasts Have an Increased Vulnerability to BSO

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Key Words: BSO, 17 β -estradiol, Friedreich's Ataxia, mitochondria

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Abstract

Human Friedreich's ataxia skin fibroblast cells have been previously shown to be extremely sensitive to free radical damage and oxidative stress compared with wild type fibroblasts. In this study, we use L-buthionine (S,R)-sulfoximine to inhibit *de novo* glutathione synthesis, depressing an important component of the intracellular defense against oxidative stress. The effective lack of glutathione allows reactive oxygen species, produced naturally via processes such as oxidative phosphorylation, to damage key components of the cellular machinery. In this study, we confirm that at 48 hours the cell viability is significantly decreased in Friedreich's ataxia fibroblasts treated with 1mM L-buthionine (S,R)-sulfoximine, but not in normal wild type fibroblasts.

Introduction

Friedreich's ataxia (FRDA) is an autosomal recessive neurodegenerative disease, and the most common form of inherited ataxia worldwide [Bradley et al., 2000; Geoffroy et al., 1976; Harding, 1981]. Cells from FRDA patients lack sufficient quantities of the protein frataxin, resulting from a trinucleotide repeat expansion in the first intron of the FXN gene [Fujita et al., 1989; Hanauer et al., 1990]. The loss of frataxin impairs the iron sulfur centers in specific energy-generating mitochondrial proteins, disrupts the cellular redox state and leads to general mitochondrial damage, ultimately resulting in cell, organ and tissue dysfunction and death [Chantrel-Groussard et al., 2001; Dürr et al., 1996; Rötig et al., 1997]. It has been shown previously that several different FRDA fibroblast cell lines are significantly more vulnerable to L-buthionine (S,R)-sulfoximine (BSO)-induced stress than age matched fibroblasts [Jauslin et al., 2002]. In this model, BSO inhibits gamma-glutamylcystein synthetase, the enzyme that joins cysteine and glutamate together into gamma-glutamylcysteine, the first step and rate limiting enzyme in the production of glutathione (GSH). This loss of GSH further impairs the ability of the FRDA cells to manage oxidative stress produced through normal cellular processes, including oxidative phosphorylation, resulting in cell death [Dürr et al., 1996]. Since normal fibroblasts do not have any frataxin level abnormality, they are relatively unaffected by the BSO-induced decrease in GSH, and at 48 hours their cell viability is not significantly decreased [Jauslin et al., 2002].

In this study, we repeat the initial experiments of Jauslin et al. [2002] to verify that our FRDA fibroblasts are affected by BSO at 48 hours after administration, while the normal fibroblasts are not. This serves as a validation of the FRDA fibroblasts as a

cellular model for FRDA and confirms a previously determined underlying difference between FRDA and normal fibroblasts [Jauslin et al., 2002, 2003].

Methods

Cell Culture. Fibroblasts from a 30 year old FRDA patient and those from an age-matched control, both obtained from Coriell Institute (Camden, NJ, USA) were maintained in Dulbecco's Modified Eagle Medium (DMEM; ThermoScientific, Waltham, MA, USA) with 10% charcoal-stripped fetal bovine serum (FBS; ThermoScientific), 1% GlutaMAX (ThermoScientific) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C, 5% CO₂ and 90% humidity. The cells were then treated for 48 hours with either dimethyl sulfoxide vehicle control (DMSO; Sigma-Aldrich, St Louis, MO, USA) or 1mM BSO. All experiments were conducted with FRDA cells from passage 15-17 and normal fibroblast cells from passage 16-18.

Calcein AM Cell Imaging and Cell Viability. Cells were plated on a 96-well plate at a density of 3,000 cells per well, then treated with vehicle or 1mM BSO. After 48 hours of BSO treatment, the media was removed, and 1 µg/mL Calcein AM (CalBiochem, San Diego, CA, USA) in phosphate buffer pH 7.2 (PBS; Fisher Scientific, Pittsburg, PA, USA) was added to each well and the plate was incubated for 10 minutes at 37°C. The cells were then photographed using a Zeiss Axio Observer Z1 inverted microscope (Carl Zeiss MicroImaging, Thornwood, NY). Cell viability was determined with a Tecan Infinite M200 plate reader with an excitation of 490nm and emission of 520nm at 48 hours.

Results

Effects of BSO on cell viability FRDA fibroblasts. Here, we investigated the relative vulnerability of human donor FRDA and normal fibroblasts to the effects of GSH depletion. We used 1 mM BSO for 48 hours to inhibit the rate limiting enzyme in the *de novo* synthesis of GSH, γ -glutamyl cysteine synthase, a concentration that we had previously determined would significantly reduce cell viability in FRDA fibroblasts [Richardson et al., 2011]. There was a significant reduction in cell viability of FRDA fibroblasts in the presence of BSO, but not in normal fibroblasts. Viability was reduced from $97.4 \pm 3.9\%$ in the vehicle control to $50.4 \pm 0.0\%$ in the BSO treated FRDA fibroblasts, compared with a non-significant reduction of $100 \pm 4.0\%$ in the vehicle control to $87.2 \pm 6.0\%$ in the BSO treated normal fibroblasts (Fig. 1). In addition, we demonstrated visually the significant reduction in the BSO treated FRDA fibroblasts compared with the other three groups via Calcein AM stained pictomicrographs (Fig. 2).

Figure Legends

Figure 1. Effects of BSO on cell viability of FRDA and normal fibroblasts. BSO concentration was 1mM. Depicted are mean \pm SD for n= 8 per group. * indicated $p<0.05$ versus vehicle control.

Figure 2. Calcein AM stained cells visually showing the effects of BSO on cell viability of FRDA and normal fibroblasts. Scale bar = 200 μ m.

Figure 1

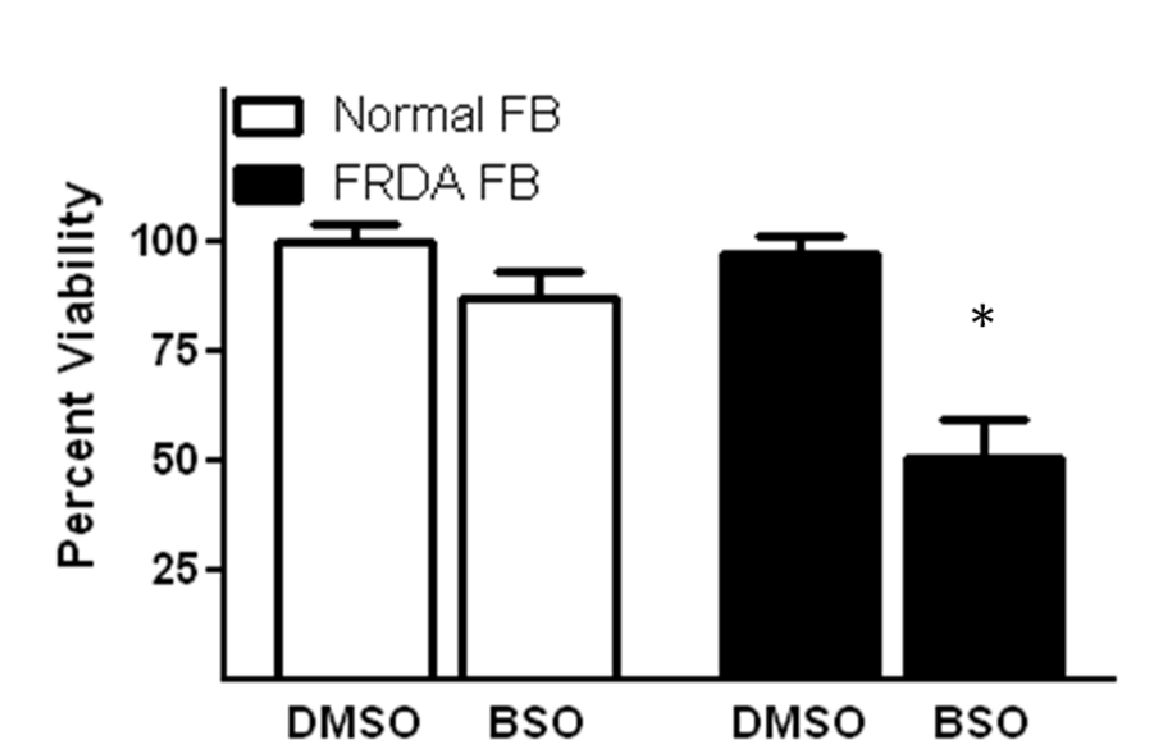
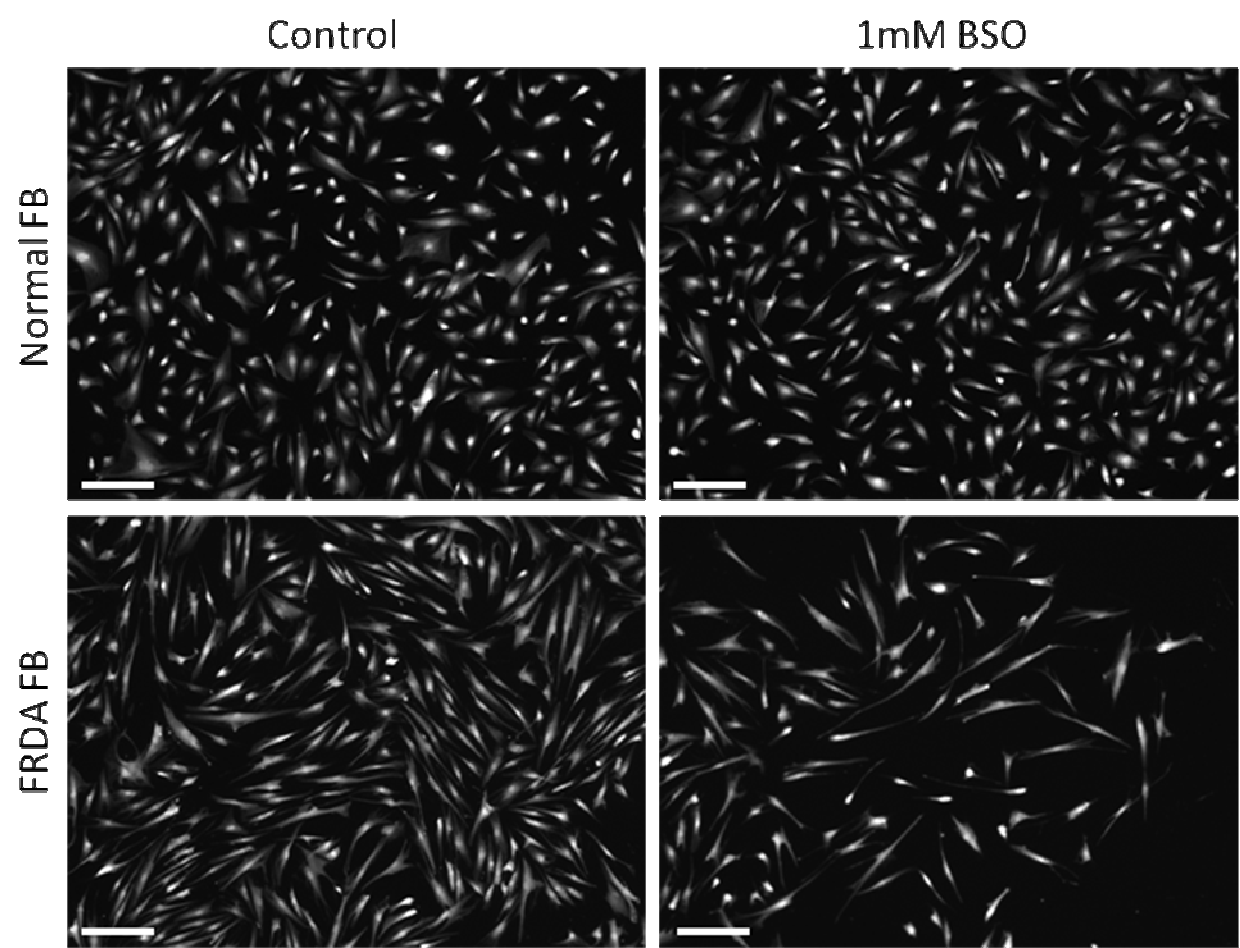


Figure 2



APPENDIX II

Estrogen Does Not Significantly Increase the Level of Frataxin Protein

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Key Words: BSO, 17 β -estradiol, Friedreich's Ataxia, mitochondria

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Abstract

Estrogen has been shown to have cytoprotective effects on a wide range of different *in vitro* and *in vivo* models, including a human Friedreich's ataxia cell model. Some of these models have been shown to act via ER α or ER β to increase transcription of various gene products crucial to cell survival, while other studies have shown estrogen to act extra-genomically and independent of any known estrogen receptor. Although our previous results indicate that estrogen protects these cells without interacting with any estrogen receptors in this cell model, it is possible that the protective effects are due to an estrogen-mediated increase in the levels of frataxin protein. In the present study, we use western blotting to determine if there is any rise in the levels of frataxin protein in these cells subsequent to estrogen administration.

Introduction

At its core, Friedreich's ataxia (FRDA) is a genetic disease resulting from the absence of intracellular frataxin protein [Bradley et al., 2000; Campuzano et al., 1996; Harding, 1983; Lodi et al., 2006; Montermini et al., 1997] due to a trinucleotide repeat expansion in the first intron of the FXN gene, effectively preventing its transcription. While the role of frataxin is not currently certain, it is involved with iron regulation and iron delivery to mitochondrial proteins containing an iron-sulfur (Fe-S) cluster [Delatycki et al., 2000; Gakh et al., 2006; Lodi et al., 2006; Santos et al., 2010]. As a result of the lack of this protein, there are a host of mitochondrial defects, including impaired Fe-S protein assembly and function such as aconitase and mitochondrial oxidative phosphorylation complexes and an overall impairment of the redox capabilities, leading to widespread cellular damage [Babcock et al., 1997; Lodi et al., 2006; Santos et al., 2010]. For this reason, many studies have focused on antioxidants to correct the redox state and prevent oxidative damage [Jauslin et al., 2002, 2003, 2007; Richardson et al., 2011, 2012] or raise the levels of frataxin to functional levels. There has been some success increasing frataxin levels using histone deacetylase inhibitors [Herman et al., 2006], erythropoietin (EPO) [Acquaviva et al., 2008; Sturm et al., 2005], and compounds that bind directly to the GAA repeats and increase transcription [Burnett et al., 2006; Grabczyk and Usdin, 2000a].

Estrogens have been found to be potently protective in FRDA fibroblasts, attenuating reactive oxygen species (ROS) [Richardson et al., 2011] and preventing lipid peroxidation, aconitase damage, mitochondrial function impairment and mitochondrial membrane potential collapse, as well as maintaining ATP production [Richardson et al.,

2012]. Our working hypothesis has been that phenolic estrogens acts as direct antioxidants, attenuating ROS and preventing their downstream effects. It is possible, however, that the mechanism of estrogen action in this cell models is to increase the levels of frataxin, thereby preventing cell death. This is unlikely, since these cells do not contain appreciable amounts of ER α or ER β [Richardson et al., 2012] and the protective effects of estrogen are not inhibited by ER antagonists, such as ICI 182,780. In this study, we use western blots to rule out the possibility that estrogens simply enhance the transcription of functional frataxin protein, adding more weight to the idea that estrogens are acting non-genomically to prevent oxidative damage in the human FRDA fibroblast model.

Methods

Cell Culture. Fibroblasts from a 30 year old FRDA patient, obtained from Coriell Institute (Camden, NJ, USA) were maintained in Dulbecco's Modified Eagle Medium (DMEM; ThermoScientific, Waltham, MA, USA) with 10% charcoal-stripped fetal bovine serum (FBS; ThermoScientific), 1% GlutaMAX (ThermoScientific) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C, 5% CO₂ and 90% humidity. Experiments were conducted with cells from passage number 16.

Steroid Treatment. FRDA fibroblasts were plated in 10 cm plates uniformly in DMEM with 10% FBS, 1% GlutaMAX and 1% penicillin-streptomycin and grown until ~80% confluent with media replaced every three days. 100nM 17 β -Estradiol (E2) or 0.1% DMSO control was added to each of the wells and two plates of the FRDA fibroblast cells were grown each for 0, 1, 2, 3, 6, 12, 24 and 48 hours, then removed using a rubber scraper and sonicated in RIPA lysis buffer. 17 β -Estradiol used in this trial was obtained from Steraloids, Inc. (Newport, RI, USA).

Western Blots. A Lowry assay was run to determine protein concentration for normalization and 20 μ g of protein was loaded into each western blot well, with human brain tissue used as the control, followed by cells treated with estrogen for 0, 1, 2, 3, 6, 12, 24 and 48 hours. Frataxin was detected using a mouse monoclonal anti-frataxin antibody (18A5DB1) obtained from Abcam MitoSciences (Abcam, Inc., Cambridge, MA, USA) at a 1:400 dilution. GAPDH (6C5) mouse monoclonal IgG antibody, obtained

from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA),
was used as a control to ensure equivalent loading of protein loaded into each well.

Results

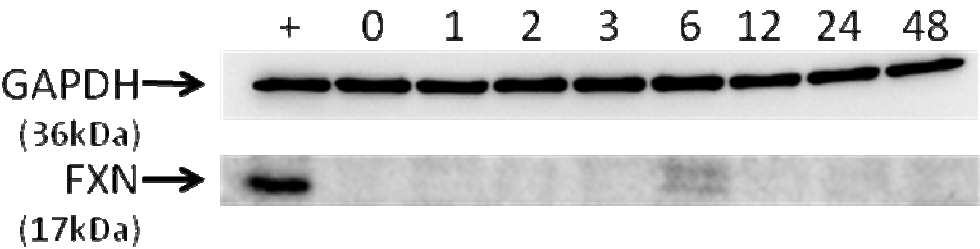
The intracellular levels of frataxin are not statistically increased by estrogen

administration. To determine the presence or absence of frataxin in the human FRDA fibroblast cell type, western blots were run confirming the absence of this protein compared to the positive control human brain sample (+). There is little to no induction of frataxin expression by estrogen at any of the time points: 0, 1, 2, 3, 6, 12, 24 or 48 hours (Fig. 1), indicating that the mechanism of action for estrogen in this disease model is unrelated to the intracellular levels of frataxin. This agrees with our previous data showing that estrogen is not inhibited by ICI 182,780 [Richardson et al., 2011] and that these cells do not express significant quantities of ER α or ER β [Richardson et al., 2012] and provides further evidence for our hypothesis that estrogen is acting via nongenomic means, likely as an antioxidant in this model.

Figure Legends

Figure 1. Western blot showing the relative absence of frataxin protein (FXN) in FRDA fibroblasts at 0, 1, 2, 3, 6, 12, 24 and 48 hours post estrogen administration compared with a positive control (+) that consisted of human brain tissue. Also shown are GAPDH markers to ensure equal protein loading in each of the wells.

Figure 1



APPENDIX III

ER β Agonists in Phase II Clinical Trials Protect Friedreich's Ataxia Fibroblasts Against BSO-Induced Oxidative Stress

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Abstract

We have previously shown that estrogens have very potent cytoprotective effects in the Friedreich's ataxia fibroblast cell model, including DPN, an estrogen receptor β preferring agonist. The present study examines the protective potential of other estrogen receptor β agonists and selective estrogen receptor modulators that have already been used in clinical trials for other indications. Here we confirm our previous observation that DPN is protective against a 1mM L-buthionine (S,R)-sulfoximine induced oxidative insult at 100nM, and show that the other biphenolic compounds, LY500307 and raloxifene are potently protective in this model, while the non-phenolic compound tamoxifen is not. These data show that LY500307, which has already qualified as a safe drug is a possible candidate drug for the treatment and prevention of Friedreich's ataxia.

Introduction

Friedreich's ataxia (FRDA) is a disease primarily of the mitochondria. Caused by a genetic defect in frataxin production [Bradley et al., 2000; Campuzano et al., 1996; Lodi et al., 2006; Pandolfo, 1998], there is insufficient delivery of iron to iron-sulfur (Fe-S) containing proteins such as heme, aconitase and electron transport chain complexes, impairing their formation and function [Babcock et al., 1997; Santos et al., 2010]. In addition, frataxin is also partially responsible for binding up free mitochondrial iron and helping to maintain the intracellular redox state [Delatycki et al., 2000; Gakh et al., 2006; Lodi et al., 2006]. Fe-S complex containing proteins, specifically aconitase, are also damaged by reactive oxygen species (ROS) in the cell [Al-Mahdawi et al., 1997; Bulteau et al., 2004; Chantrel-Groussard et al., 2001; Gakh et al., 2006], and so their function is further impaired by the ROS build up produced by the lack of frataxin. The oxidative damage seen in FRDA to the mitochondria is cumulative and progressive [Karthikeyan et al., 2003], resulting in a mismatch between energy demand and energy production ending in cell death. This occurs first in the tissues that are most dependent on oxidative phosphorylation to produce ATP and have the highest amount of frataxin production in normal individuals [Al-Mahdawi et al., 2006; Dürr et al., 1996; Dutka et al., 2000], such as the heart and spinal cord, although it is still unknown why certain ascending spinal cord tracts are affected while other areas of the brain and spinal cord are virtually undamaged [Santos et al., 2010].

Because of the contribution of the redox state to the affected cells, several studies have focused on antioxidants as a potential therapy [Jauslin et al., 2002, 2003, 2007], recently including phenolic estrogens [Richardson et al., 2011, 2012]. Because of the

problems with hormone therapy illustrated by the Women's Health Initiative much of the translational research involving estrogens has been put on hold [Simpkins and Singh, 2008], making it difficult for many estrogen-like compounds to be viewed as viable candidate drugs. For this reason, much of the research in recent years has focused on non-feminizing estrogens. There have been several types of estrogen-related drugs that have been successfully brought into the clinic, including the selective estrogen receptor modulators (SERMs). One particular compound, LY500307, a Benzopyran selective estrogen receptor beta agonist (SERBA) has been shown to be selective at ER β in CD-1 mice and showed beneficial effects in benign prostatic hyperplasia (BPH) in this model [Norman et al., 2006]. The phase II clinical trial on LY500307 was stopped in October 2011 due to a lack of efficacy in treating BPH [<http://clinicaltrials.gov/ct2/show/NCT01097707>], however it passed a phase I clinical trial, indicating that it is a reasonably safe and well tolerated compound for humans. LY500307 is a biphenolic drug, similar to our previously tested DPN compound [Richardson et al., 2011], and likely to show similar potency and efficacy in terms of preventing cell death in our human FRDA fibroblast model. Since this drug has been already evaluated as safe for humans, it is an excellent candidate drug for consideration for treating FRDA.

In this study, we evaluate the potential for LY500307, raloxifene and tamoxifen compared to DPN to protect FRDA fibroblasts from a BSO-induced oxidative insult in terms of cell viability. We show that the phenol ring containing compounds DPN, LY500307, and raloxifene are all capable of preventing much of the cell death associated with BSO treatment, while the non-phenolic tamoxifen is not. These data indicate that

LY500307 is a very efficacious compound in preventing oxidative damage from killing FRDA fibroblasts, and since it has already been approved as safe in a phase I clinical trial, it is a good candidate drug for further study for prevention of FRDA pathology and symptoms.

Methods

Cell Culture. Fibroblasts from a 30 year old FRDA patient, obtained from Coriell Institute (Camden, NJ, USA) were maintained in Dulbecco's Modified Eagle Medium (DMEM; ThermoScientific, Waltham, MA, USA) with 10% charcoal-stripped fetal bovine serum (FBS; ThermoScientific), 1% GlutaMAX (ThermoScientific) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C, 5% CO₂ and 90% humidity. Before vehicle or BSO treatment, FRDA fibroblast media was changed to phenol red- and sodium pyruvate-free DMEM (ThermoScientific) containing 1% penicillin-streptomycin. All experiments were conducted with FRDA cells from passage 15-18.

Chemicals & Reagents. L-buthionine (S,R)-sulfoximine (BSO) was acquired from Sigma-Aldrich (St Louis, MO, USA). Diarylpropionitrile (DPN) was purchased from Tocris Bioscience (Ellisville, MO, USA). LY500307, raloxifene and tamoxifen were purchased from Selleck Chemicals (Houston, TX, USA). Structures for these steroids are provided in Fig. 1.

Steroid Treatment. FRDA fibroblasts were plated in 24- or 96-well plates at a density of 3,000 or 5,000 cells per well in DMEM with 10% FBS, 1% GlutaMAX and 1% penicillin-streptomycin, depending on the assay. After 24 hours the growth media was removed and replaced with the phenol red- and sodium pyruvate-free DMEM. The cells were then treated for 48 hours, depending on the assay, with either dimethyl sulfoxide vehicle control (DMSO; Sigma-Aldrich, St Louis, MO, USA) or 1mM BSO in the

presence of 1 nM-10 μ M DPN, LY500307, raloxifene or tamoxifen, concentrations of estrogen-like molecules which have been shown to be neuroprotective in various cell lines [Yi et al., 2008] and cytoprotective in this FRDA fibroblast line [Richardson et al., 2011].

Calcein AM Cell Viability Assay. Cells were plated on a 96-well plate at a density of 3,000 cells per well, then treated with vehicle or 1mM BSO and simultaneous DPN, LY500307, raloxifene or tamoxifen treatment. After 48 hours of BSO treatment, the media was removed, and 1 μ g/mL Calcein AM (CalBiochem, San Diego, CA, USA) in phosphate buffer pH 7.2 (PBS; Fisher Scientific, Pittsburg, PA, USA) was added to each well and the plate was incubated for 10 minutes at 37°C. Cell viability was determined with a Tecan Infinite M200 (Tecan Systems, Inc., San Jose, CA) plate reader with an excitation of 490nm and emission of 520nm at 48 hours.

Calcein AM Cell Imaging. Cells were plated on a 96-well plate at a density of 5,000 cells per well, then treated with vehicle or 1mM BSO and DPN, LY500307, raloxifene or tamoxifen. After 48 hours of BSO treatment, the media was removed, and 1 μ g/mL Calcein AM (CalBiochem) in phosphate buffer pH 7.2 (PBS; Fisher Scientific) was added to each well and the plate was incubated for 10 minutes at 37°C. The cells were then photographed using a Zeiss Axio Observer Z1 inverted microscope (Carl Zeiss MicroImaging, Thornwood, NY).

Results

The effects of LY500307 on cell viability in BSO-treated FRDA fibroblasts.

To determine if LY500307 had a protective effect countering the oxidative damage caused by glutathione depletion allowed by BSO treatment, we tested 1 pM-1 μ M LY in cells treated with 1mM BSO (Fig. 2). LY500307 had a significant protective effect ($p<0.05$) at 100 nM, 1 μ M and 10 μ M ($n=8$), although it seemed to exhibit a toxic effect beginning at 1 μ M-10 μ M. The EC_{50} of LY500307 was at 65.2 nM, with near maximal effects at 100 nM. Since these cells lack significant quantities of ER α or ER β [Richardson et al., 2012], it can be concluded that LY500307, an ER β selective agonist, is acting independently of these receptors in this model, likely by direct antioxidant effects provided by the phenol rings in its structure, similar to our previous observations [Richardson et al., 2011, 2012].

The effects of a single dose of DPN, LY500307, raloxifene and tamoxifen on cell viability in BSO-treated FRDA fibroblasts. To compare the effects of LY500307 to the previously tested DPN [Richardson et al., 2011] and other well known SERM compounds raloxifene and tamoxifene, we tested the dose of LY500307 that produced maximum efficacy, 100 nM, of each compound with a Calcein AM assay (Fig. 3). There was no statistically significant difference between DPN, LY500307 and raloxifene, the three biphenolic compounds assayed at 100nM. All three provided significant protection against the BSO-induced oxidative insult. Tamoxifen, the non-phenolic compound, provided no significant protection and the measured cell viability was not significantly different from the BSO alone treated cells. This again provides evidence that it is the phenol rings providing antioxidant related protection, not ER binding ability that is

responsible for the protection seen in LY500307 and other estrogen like compounds.

Calcein AM images visually showing the protection provided by DPN, LY500307 and raloxifene, but not tamoxifen are also shown (Fig. 4).

Discussion

FRDA is a devastating neurological disease that results in degeneration of the dorsal root ganglia (DRG), progressive gait ataxia, tremor, weakness, sensory abnormalities, pes cavitus, lateral and kyphoscoliosis, type 1 diabetes and cardiac abnormalities, including hypertrophic cardiomyopathy, the leading cause of premature death in these patients [Al-Mahdawi et al., 2006; Dürr et al., 1996; Dutka et al., 2000; Geoffroy et al., 1976; Harding, 1981; Harding, 1983; Isnard et al., 1997; Lodi et al., 2006]. With an incidence of 1:50,000-1:20,000 and a carrier rate of 1:120-1:60 in the United States Caucasian population, it is the most common form of inherited ataxia worldwide [Harding, 1983]. As yet, there is very little successful treatment for the disorder [Lodi et al., 2006; Santos et al., 2010]. Much of the research thus far has focused on the redox state within the affected cells, leading to the development and use of novel antioxidants targeting the mitochondria, aimed at preventing damage to this organelle and preventing cell death [Jauslin et al., 2002, 2003, 2007]. Recently, our lab has proposed the use of phenol ring containing estrogens as a viable antioxidant based on the successful prevention of cell death in a human FRDA fibroblast cell line treated with BSO [Richardson et al., 2011].

This study examines the initial viability of compounds already approved as safe for human use for other indications [<http://clinicaltrials.gov/ct2/show/NCT01097707>] for use as FRDA therapies. We show that a dose of 100 nM, LY500307 is able to prevent cell death at a level comparable to other estrogens [Richardson et al., 2011] and other novel antioxidants synthesized specifically to treat this disorder [Jauslin et al., 2002, 2003, 2007]. Since FRDA is genetically present at birth, but symptoms do not arise until

early childhood, there is a window of time to begin treatment with an antioxidant to prevent cell death and symptom development. This time frame is ideal to treat with a non-feminizing estrogen, as this would allow for normal endocrine development but possibly delay or even prevent the devastating symptoms and premature death seen in FRDA patients.

Figure Legends

Figure 1. Structures of compounds assessed for protection against BSO toxicity in FRDA fibroblasts.

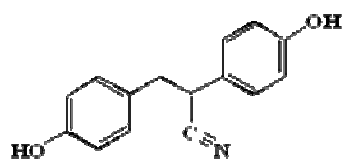
Figure 2. Dose response curve of 1 pM-10 μ M LY500307 in the presence of 1 mM BSO. Depicted are mean \pm SD for n= 8 per group. * indicated $p<0.05$ versus BSO alone-treated cells.

Figure 3. Effects of DPN, LY500307, Raloxifene and Tamoxifen on cell viability in BSO-treated FRDA fibroblasts. BSO concentration was 1 mM and all steroid concentrations were 100 nM. Depicted are mean \pm SD for n= 8 per group. * indicated $p<0.05$ versus BSO alone-treated cells.

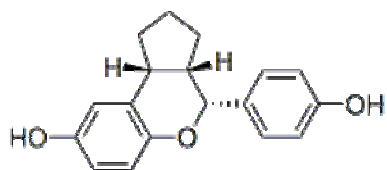
Figure 4. Calcein AM stained cells visually showing the effects of DPN, LY500307, Raloxifene and Tamoxifen on cell viability in BSO-treated FRDA fibroblasts. BSO concentration was 1 mM and all steroid concentrations were 100 nM. Scale bar = 200 μ m.

Figure 1

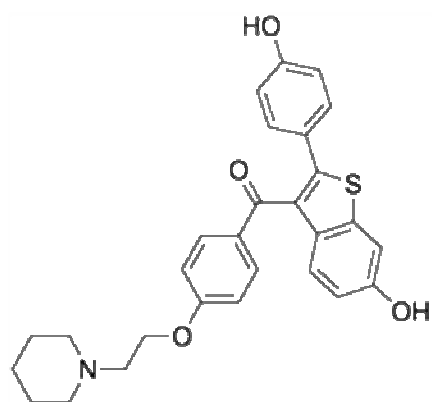
DPN



LY500307



Raloxifene



Tamoxifen

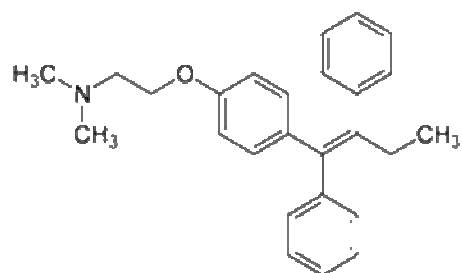


Figure 2

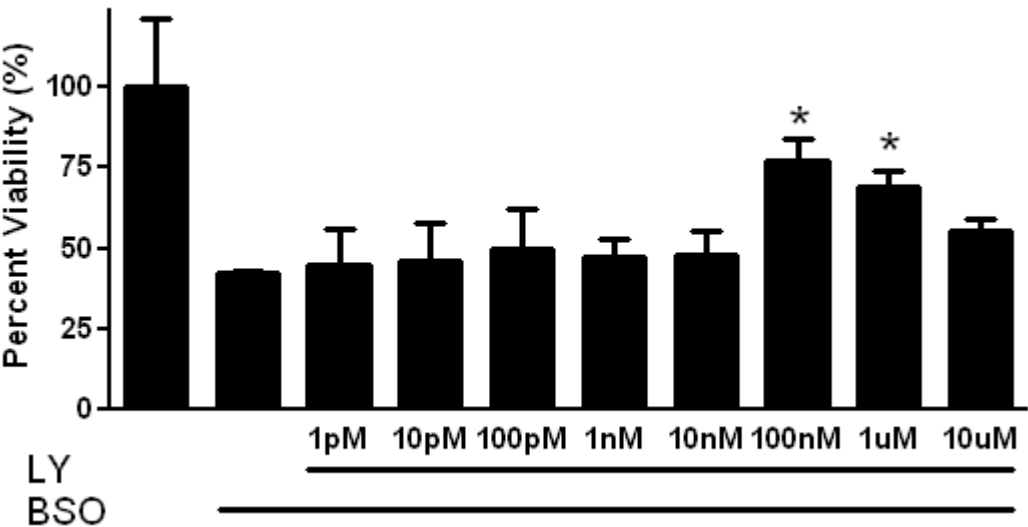


Figure 3

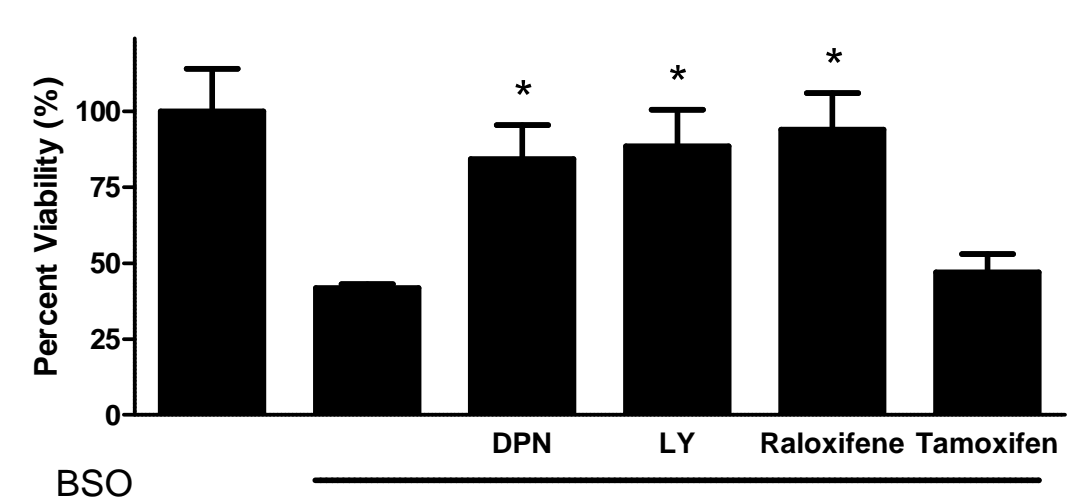
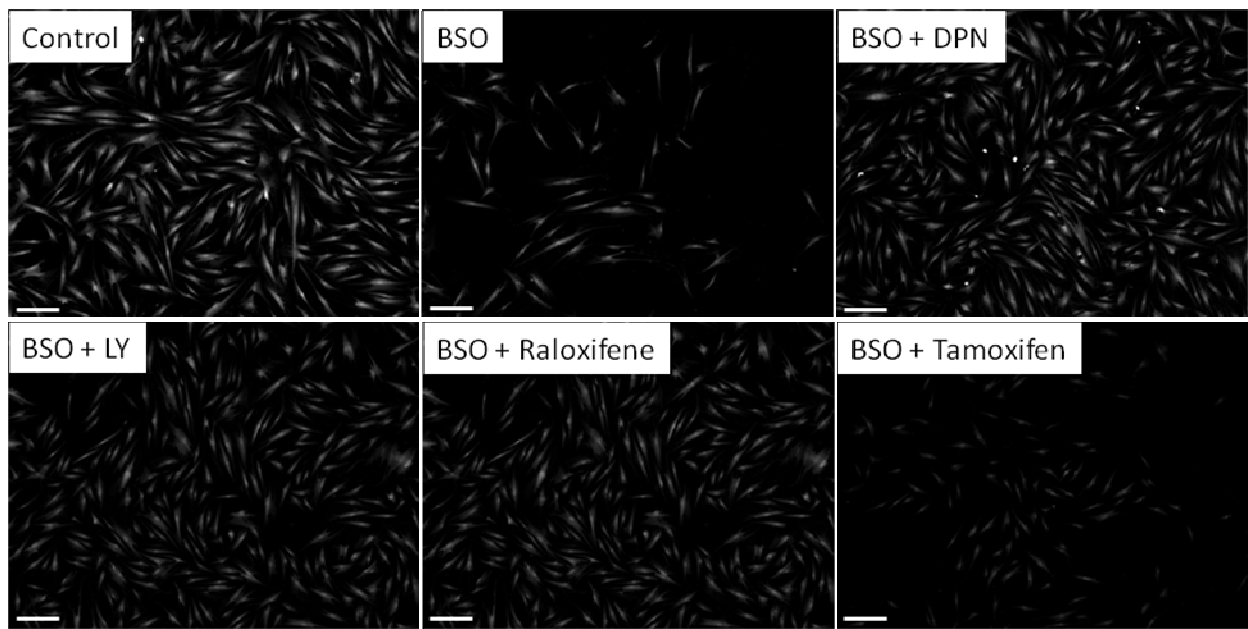


Figure 4



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<http://clinicaltrials.gov/ct2/show/NCT01097707>

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