Iron-Induced Cardiac Damage: Role of Apoptosis and Deferasirox Intervention

Yeling Wang, Miaozong Wu, Rabaa Al-Rousan, Hua Liu, Jacqueline Fannin, Satyanarayana Paturi, Ravi Kumar Arvapalli, Anjaiah Katta, Sunil K. Kakarla, Kevin M. Rice, William E. Triest, and Eric R. Blough


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ABSTRACT
Excess cardiac iron levels are associated with cardiac damage and can result in increased morbidity and mortality. Here, we hypothesize that elevations in tissue iron can activate caspase-dependent signaling, which leads to increased cardiac apoptosis and fibrosis, and that these alterations can be attenuated by iron chelation. Using an iron-overloaded gerbil model, we show that increased cardiac iron is associated with reduced activation of Akt (Ser473 and Thr308), diminished phosphorylation of the proapoptotic regulator Bad (Ser136), and an increased Bax/Bcl-2 ratio. These iron-overload-induced alterations in Akt/Bad phosphorylation and Bax/Bcl-2 ratio were coupled with increased activation of the downstream caspase-9 (40/38- and 17-kDa fragments) and apoptosis executioner caspase-3 (19- and 17-kDa fragments), which were accompanied by evidence of elevated cytoskeletal α-fodrin cleavage (150- and 120-kDa fragments), discontinuity of myocardial membrane dystrophin immunoreactivity, increases in the number of terminal deoxyribonucleotidyl transferase dUTP nick-end labeling (TUNEL)-positive cells (nucleic DNA fragmentation), and cardiac fibrosis. We demonstrate that the administration of deferasirox, a tridentate iron chelator, is associated with diminished tissue iron deposition, attenuated activation of caspases, reduced α-fodrin cleavage, improved membrane integrity, decreased TUNEL reactivity, and attenuated cardiac fibrosis. These results suggest that the activation of caspase-dependent signaling may play a role in the development of iron-induced cardiac apoptosis and fibrosis, and deferasirox, via a reduction in cardiac tissue iron levels, may be useful for decreasing the extent of iron-induced cardiac damage.

Introduction
Excess tissue iron content as a result of iron overload or accumulation is estimated to affect up to 100 million people worldwide. It is often seen in individuals with hereditary hemochromatosis and in those who require frequent blood transfusions, such as individuals afflicted with thalassemia, sickle cell disease, and myelodysplastic syndrome (Burke et al., 2002; Abetz et al., 2006). Excess tissue iron accumulation is toxic and can lead to heart failure, which is the major cause of death after protracted iron overload (Borgna-Pignatti et al., 2005; Kohgo et al., 2008). Although the causes of cardiomyopathy after iron overload are not fully understood, it has been suggested that iron overload is associated with increases in myocardial apoptosis and the development of fibrosis (Whittaker et al., 1996; Oudit et al., 2004; Arvapalli et al., 2010).

The factors regulating apoptosis are complicated; however, several studies have posited that caspase-3 plays a central role in the execution phase of cell apoptosis (Jänicke et al., 1998; Porter and Jänicke, 1999). The activation of caspase-3 is governed by a group of signaling cascades, among which the interaction of antiapoptotic Bcl-2 and proapoptotic Bax proteins plays a critical role. Bcl-2 is capable of forming a
heterodimer with Bax, thereby preventing Bax homodimerization, the release of mitochondria cytochrome c, and activation of protease caspase-9 and, subsequently, caspase-3 (Gross et al., 1998; Narita et al., 1998; Murphy et al., 2000). In addition to Bax/Bcl-2 signaling, activation of caspase-mediated proteolytic cascade can be regulated by another proapoptotic factor, Bad. Bad can affect the binding of Bax to Bcl-2 through its ability to bind to Bcl-2 (Yang et al., 1995). The ability of Bad to bind to Bcl-2 is believed to be abrogated when Bad becomes phosphorylated at Ser136 by Akt/protein kinase B, a serine/threonine-specific protein kinase that is involved in regulating cell survival (Zha et al., 1996; Datta et al., 1997; Wu et al., 2010b). Whether iron overload affects the regulation of these caspase-mediated proteolytic cascades is currently unclear.

The initial treatment for the accumulation of excess iron is typically pharmacological intervention with deferoxamine or deferiprone. Although effective, treatment compliance is frequently compromised because the administration of these compounds is often burdensome or associated with serious side effects (Neufeld, 2006). Research over the last few years has suggested that once-daily treatment with deferasirox (Exjade (ICL 670, C21H15N3O4); Novartis Pharmaceuticals, East Hanover, NJ), an orally administered tridentate iron chelator, may be effective for the treatment of iron overload (Neufeld, 2006; Al-Rousan et al., 2009; Arvapalli et al., 2010; Pennell et al., 2010). Although initial findings regarding the use of deferasirox have demonstrated effectiveness in removing cardiac iron in animals and humans, whether this agent is capable of diminishing iron-associated increases in cardiomyocyte apoptosis and fibrosis has not been investigated.

Our previous studies using the Mongolian gerbil model have demonstrated that deferasirox treatment is capable of reducing cardiac iron content after iron overload (Al-Rousan et al., 2009). Using another subset of animals and tissues from the same groups (Al-Rousan et al., 2009), we hypothesize that excess tissue iron can activate caspase-dependent signaling, which leads to increased cardiac apoptosis and fibrosis, and that these alterations can be attenuated by iron chelation. Our findings demonstrate that iron overload in the heart is associated with increased activation of caspase-3, elevated α-fodrin cleavage, and discontinuous myocardial membrane, along with increased nuclear DNA fragmentation and cardiac fibrosis, and deferasirox treatment is effective in attenuating iron-overload associated cardiac damage.

Materials and Methods

Materials. Primary antibodies against caspase-3, caspase-9, α-fodrin, Bcl-2, Bax, Akt, phospho-Akt (Thr308), phospho-Akt (Ser473), Bad, phospho-Bad (Ser136), glyceraldehyde-3-phosphate dehydrogenase, and rabbit secondary antibodies (anti-rabbit) were purchased from Cell Signaling Technology (Danvers, MA). Ferritin heavy chain (H-53) antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Ferritin light chain antibodies were procured from Alpha Diagnostic International Inc. (San Antonio, TX). The dextran phosphate antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Ferritin light chain antibodies were procured from Alpha Diagnostic International Inc. (San Antonio, TX). The dystrophin antibody was obtained from Novocastra/Vector Laboratories (Burlingame, CA), and the in situ cell death detection kit was obtained from Roche Diagnostics (Mannheim, Germany). VECTASHIELD HardSet Mounting medium with DAPI was obtained from Vector Laboratories (Burlingame, CA). Picrosirius red was obtained from R&D Systems (Minneapolis, MN) and collected on polylysine-coated slides. DNA fragmentation was detected using a terminal deoxyribonucleotide transferase-mediated dUTP nick end-labeling (TUNEL) assay. TUNEL, dystrophin, and DAPI triple staining were performed as described previously (Wu et al., 2009b; Arvapalli et al., 2010; Kakarla et al., 2010).

Measurement of Collagen Content. Picrosirius red staining was used to measure interstitial collagen on heart sections as described elsewhere (Dolber and Spach, 1987). In brief, tissue sections were fixed with 95% alcohol for 1 min, washed three times with tap water, and then stained with hematoxylin for 8 min and picrosirius red for 20 min. After dehydration sections and mounting, collagen content was determined from 15 randomly selected regions from each tissue section at 40× magnification using an Olympus BX51 microscope (Olympus, Center Valley, PA). Collagen area was measured using the ImageJ software (http://rsweb.nih.gov/ij/) as outlined previously (Wu et al., 2009b).

Perls Iron Staining. Tissue iron distribution was determined via a Perls iron staining according to the manufacturer’s protocol (Sigma-Aldrich). The area of iron deposition was quantified with the use of ImageJ software.

Immunoblotting Analysis. Tissue protein was extracted via homogenization in tissue protein extraction reagent buffer containing protease and phosphatase inhibitors as detailed previously (Wu et al., 2009a,b, 2010a). Protein concentration of homogenates was determined via the Bradford (1976) method. Forty micrograms of protein was boiled in SDS-loading buffer and separated on 10 or 15% SDS-polyacrylamide gel electrophoresis before being transferred to nitrocellulose membranes as described previously (Wu et al., 2009a,b, 2010a; Arvapalli et al., 2010). Gels were stained with RAPID protein reagent to confirm equal protein loading and the
transfer efficiency of proteins onto membrane. Antigens of interest were visualized after incubation of membranes with specific antibodies and development with an ECL reagent. Target protein levels were quantified by AlphaEaseFC image analysis software (Alpha Innotech, San Leandro, CA) and normalized to the amount of glyceraldehyde-3-phosphate dehydrogenase.

Data Analysis. The results are presented as mean ± S.E. Data were analyzed using SigmaStat 3.5 software (Systat Software, Inc., San Jose, CA). The effects of iron and the role of deferasirox were evaluated by analysis of variance, followed by a Student-Newman-Keuls test for multiple-comparison testing. Values of P < 0.05 were considered statistically significant.

Results

Deferasirox Treatment Reduces Cardiac Iron Deposition. As detailed previously (Al-Rousan et al., 2009), inductively coupled plasma-atomic emission spectroscopy (ICP-AES) demonstrated that cardiac iron content was 10.4-fold higher after 10 weeks of iron overload (IO) and 7.4-fold higher after 3 months of follow-up (IO-3m). Once-daily treatment with deferasirox significantly decreased overload-induced cardiac iron levels (Al-Rousan et al., 2009). Perls staining of cardiac sections was used to expand on these findings and demonstrated that iron was deposited both within and outside cardiomyocytes of the iron-overloaded gerbil heart (Fig. 1A). Consistent with our ICP-AES data, iron overload significantly increased the area of cardiac tissue that reacted positively for iron (P < 0.05). This finding persisted in the 3-month follow-up animals (P < 0.05) but was significantly reduced after deferasirox treatment (P < 0.05; Fig. 1A). Supporting our finding of increased tissue iron, iron overload significantly increased the amount of ferritin heavy and light chains, two key regulators of iron metabolism and storage (Kohgo et al., 2008; Arvapalli et al., 2010), both after loading and after 3 months of follow-up (P < 0.05). Consistent with our measurement of tissue iron, deferasirox treatment decreased the abundance of ferritin heavy and light chains (P < 0.05; Fig. 1B).

Deferasirox Diminishes Iron Overload-Induced Myocardial Fibrosis. To examine the potential effects of increased tissue iron on cardiac fibrosis, picrosirius red staining was used to semiquantitatively assess collagen accumulation. Iron overload significantly increased collagen deposition, whether examined immediately after the loading period or after the 3-month follow-up period (P < 0.05; Fig. 2). Compared with age-matched controls, deferasirox treatment significantly decreased cardiac collagen accumulation (−54%, P < 0.05; Fig. 2).

Deferasirox Decreases Iron Overload-Induced Cardiomyocyte Nucleic DNA Fragmentation. To investigate the possibility that tissue iron accumulation was associated with an increase in cardiomyocyte apoptosis, we examined the number of nuclei staining positively for DNA fragmentation using TUNEL staining. Compared with age-matched controls, the number of TUNEL-positive nuclei was significantly increased both after iron overload and remained elevated after 3 months of follow-up (P < 0.05; Fig. 3). Deferasirox treatment was associated with a significant decrease in the number of TUNEL-reactive cells (−77%, P < 0.05; Fig. 3).

Deferasirox Decreases Iron Overload-Induced Activation of Caspasases. Caspase-3 is a critical executioner of apoptosis (Jänicke et al., 1998; Porter and Jänicke, 1999). Compared with age-matched controls, iron overload in...
increased the amount of the cleaved (activated) caspase-3 (19- and 17-kDa fragments) immediately and 3 months after iron overload (*P* ≤ 0.05; Fig. 4A). This finding was attenuated with deferasirox treatment as the amount of caspase cleavage was decreased by 51 and 53% for the 19- and 17-kDa fragments, respectively (*P* ≤ 0.05; Fig. 4A).

To confirm our finding of increased caspase-3 activation with iron overload, we next examined the activity of caspase-9, which is thought to be an upstream regulator of caspase-3 (Jänicke et al., 1998). Similar to caspase-3 data, the amount of cleaved (active) caspase-9 (40/38- and 17-kDa) was significantly increased after iron overload and 3 months of follow-up (*P* < 0.05; Fig. 4B). Compared with age-matched controls, deferasirox treatment significantly decreased the iron overload-induced activation of caspase-9 by 51 and 53% for the 40/38- and 17-kDa fragments, respectively (*P* < 0.05; Fig. 4B).

Next, we investigated whether iron-induced caspase-3 activation was associated with increased cleavage of α-fodrin. The α-fodrin molecule was chosen on the basis of previous reports suggesting that it is a substrate of caspase-3 and because it plays a critical role in stabilizing the cell membrane (Martin et al., 1995; Jänicke et al., 1998; Kakarla et al., 2010). Consistent with our findings of increased caspase-3 activation, the amount of cleaved α-fodrin (150- and 120-kDa) was significantly increased after iron overload and after 3 months of follow-up (*P* < 0.05; Fig. 4C). Deferasirox treatment decreased iron overload-induced fragmentation of α-fodrin by 56 and 52% for the 150- and 120-kDa fragments, respectively (*P* < 0.05; Fig. 4C).

Because the cleavage of α-fodrin has been linked to increases in membrane blebbing and the development of apoptosis (Martin et al., 1995; Jänicke et al., 1998), we examined whether increased α-fodrin fragmentation was associated with alterations in dystrophin localization. Immunohistochemical staining demonstrated a discontinuity of myocardial membrane dystrophin immunoreactivity after iron overload and in the animals 3 months after follow-up, whereas tissue sections obtained from deferasirox-treated animals tended to resemble more closely those from age-matched and non–iron-overloaded control animals (Fig. 4D).

**Deferasirox Prevents Iron Overload-Induced Alterations in Caspase-Activation Signaling.** The expression of Bax, a proapoptotic protein critical for caspase-9 activation...
(Gross et al., 1998), was not different between groups \((P = 0.10; \text{Fig. 5A})\), whereas the expression of the antiapoptotic Bcl-2 protein (Murphy et al., 2000; Wu et al., 2009b) was decreased in the animals that had been subjected to 10 weeks of iron overload. Deferasirox treatment increased the amount of Bcl-2 compared with that observed in age-matched IO control animals \((+105\%, P < 0.05; \text{Fig. 5A})\). The ratio of Bax/Bcl-2, a predictive marker of mitochondria-mediated caspase-9 activation (Korsmeyer et al., 1993), was increased in animals after 10 weeks of iron overload, whereas deferasirox treatment decreased the Bax/Bcl-2 ratio compared with that observed in age-matched iron-overloaded control animals \((-56\%, P < 0.05; \text{Fig. 5A})\).

The expression of Bad, another proapoptotic protein that promotes cell death by disturbing the inhibitory effects of Bcl-2 on Bax (Yang et al., 1995), was not different between groups \((P = 0.17)\). However, phosphorylation of Bad at Ser136, which acts to inhibit the apoptotic activity of this protein, was decreased after iron overload and in the animals 3 months after follow-up \((P < 0.05)\). Deferasirox treatment significantly increased Bad (Ser136) phosphorylation compared with that of age-matched iron-overloaded animals \((P < 0.05; \text{Fig. 5B})\).

The phosphorylation of Bad at Ser136 is thought to be mediated by Akt, a kinase that functions to promote cell survival and prevent apoptosis (Zha et al., 1996; Datta et al., 1997; Wu et al., 2009b). The phosphorylation and activation of Akt at both Ser473 and Thr308 were decreased after iron overload and in the animals after 3 months of follow-up \((P < 0.05)\), whereas deferasirox treatment significantly increased phosphorylation of Akt to levels comparable with that seen in the age-matched iron-overloaded animals \((P < 0.05; \text{Fig. 5C})\).

**Discussion**

Excess tissue iron levels are toxic and can lead to myocardial injury and heart failure, one of the major causes of death in those afflicted with iron overload (Borgna-Pignatti et al., 2005; Kohgo et al., 2008). Our previous work using the iron-overloaded Mongolian gerbil has demonstrated that deferasirox is effective in reducing cardiac iron content (Al-Rousan et al., 2009). Extending on this study, we demonstrate here that increased cardiac iron is associated with increased apoptosis and fibrosis, a finding that appears to persist over time, because these alterations were also found in animals that had undergone iron loading and were then analyzed 3 months later. It is important to note that our data also demonstrate that deferasirox administration is effective in attenuating iron overload-associated cardiac apoptosis and fibrosis.
Increases in cardiac fibrosis and apoptosis have been implicated in the pathological remodeling of the heart and have been shown to be involved in the progression to heart failure (Burlew and Weber, 2002; Gurtl et al., 2009). Using picrosirius red staining to examine collagen deposition, we demonstrate that iron overload is associated with increased cardiac interstitial fibrosis and that the extent of fibrosis is decreased with deferasirox treatment (Fig. 2). Increases in cardiac apoptosis are thought to be associated with increased cleavage of cytoskeletal protein α-fodrin, loss of plasma membrane integrity, and increased DNA fragmentation (Majno and Joris, 1995; Martin et al., 1995; Jänicke et al., 1998; Zhang and Xu, 2000; Fadeel, 2004; Takada et al., 2004). Confirming this possibility, we found an increased number of cardiac myocytes exhibiting DNA strand breaks (TUNEL-positive; Fig. 3), increased α-fodrin cleavage (Fig. 4C), and discontinuity in cardiomyocyte membrane immunoreactivity for dystrophin (Fig. 4D) with iron overload. It is important to note that we also found that each of these alterations was attenuated by deferasirox treatment. In our previous study we demonstrated that 10 weeks of iron overload increased cardiac iron content as determined by ICP-AES (Al-Rousan et al., 2009). As an extension of that study, we examined here how iron overload affects the spatial deposition of iron in myocardium. Consistent with our aforementioned study, we found evidence of increased iron deposition after 10 weeks of iron overload, an observation that remains present even after 3 months of follow-up. As expected, deferasirox treatment was associated with reduced iron deposition and decreased expression of ferritin heavy and light chain molecules (Fig. 1, A and B). It is especially noteworthy that regions of iron deposition appeared to be spatially and quantitatively associated with areas of cellular apoptosis and fibrosis (Figs. 1A, 2, and 3), a finding that is consistent with the possibility that excess iron can lead to cardiac damage.

To further our understanding of how iron overload induces apoptosis, we investigated the regulation of caspase-3. As a critical executioner of apoptosis (Jänicke et al., 1998; Porter and Jänicke, 1999), caspase-3 was activated after iron overload at the 3-month follow-up time point (Fig. 4A). This activation was associated with increased activation of its upstream protease, caspase-9 (Fig. 4B). To verify the functionality of caspase-3 activation, we also examined whether elevations in tissue iron were associated with the increased cleavage of α-fodrin, a primary target of caspase-3 that has been implicated in maintenance of normal membrane structure and supporting cell surface protein function (Martin et al., 1995; Jänicke et al., 1998; Kakarla et al., 2010). We demonstrated that the 150- and 120-kDa fragments of cleaved α-fodrin were significantly increased in animals after 10 weeks of iron overload and in the animals 3 months after follow-up. It is important to note that these iron overload-associated increases in α-fodrin cleavage were significantly reduced with deferasirox treatment (Fig. 4C). To investigate whether the fragmentation of α-fodrin might affect cardiomyocyte membrane integrity, we next examined the spatial distribution of dystrophin, a critical transmembrane protein (Ervasti and Campbell, 1993; Wu et al., 2009b; Kakarla et al., 2010). It is noteworthy that tissue sections obtained from the hearts of both the iron-overloaded and 3-month follow-up...
groups exhibited discontinuity of dystrophin immunoreactivity, a finding that was substantially diminished after deferasirox treatment (Fig. 4D). Taken together, these results are consistent with the notion that iron overload is associated with activation of caspase signaling, cleavage of β-fodrin, and disruption of cardiomyocyte membrane integrity (Fig. 6).

Among the signaling molecules that have been thought to be involved in the activation of caspase-9, Bax and its regulatory partners play a critical role. Upon apoptotic stimulation, Bax stimulates the release of mitochondrial cytochrome c, which in turn is thought to activate the protease activity of caspase-9 (Korsmeyer et al., 1993; Gross et al., 1998; Narita et al., 1998; Murphy et al., 2000). It is postulated that the ratio of Bax/Bcl-2 proteins plays a critical role in regulating cellular apoptosis, with cell death being favored as the balance shifts toward Bax (Korsmeyer et al., 1993; Murphy et al., 2000). Here, we found that iron overload was associated with a reduction in the amount of Bcl-2 protein, resulting in a significant increase of the Bax/Bcl-2 ratio (Fig. 5A). As expected from our caspase data, deferasirox treatment was associated with increases in the amount of Bcl-2 expression and hence a decreased Bax/Bcl-2 ratio compared with that observed in age-matched iron-overloaded animals (Fig. 5A). These data suggest that excess tissue iron has an adverse effect on Bcl-2 expression and that deferasirox-associated reductions in caspase activation and cellular apoptosis might be related to improvements in the Bcl-2/Bax ratio. This possibility is supported by reports in the literature because other studies, performed in vitro, have shown that elevated iron can reduce Bcl-2 expression without affecting Bax levels (Carlini et al., 2006; Koocumchoo et al., 2006). Whether the reduction in Bcl-2 protein observed under conditions of elevated iron are due to transcriptional changes or protein degradation is currently unclear and will require further study.

It is well documented that the proapoptotic factor Bad can affect the binding of Bax to Bcl-2 through its ability to bind to Bcl-2 (Yang et al., 1995). It is thought that the ability of Bad to bind to Bcl-2 is abrogated when Bad becomes phosphorylated at Ser136 by Akt/protein kinase B, a serine/threonine-specific protein kinase that is involved in regulating cell survival (Zha et al., 1996; Datta et al., 1997; Wu et al., 2010b). Here, we demonstrate that the degree of Bad phosphorylation (Ser136) was decreased by iron overload, even though the expression of Bad did not change (Fig. 5B). As predicted, this reduction in Bad phosphorylation was found to be associated with reduced phosphorylation/activation of Akt (Thr308 and Ser473) (Fig. 5C). Consistent with the alteration of caspase activation and cellular apoptosis, deferasirox treatment restored this iron-induced reduction in Akt phosphorylation. It is noteworthy that this treatment-related increase in phosphorylated Akt was associated with increased phosphorylation of Bad (Fig. 5C). Taken together, these data suggest that Akt/Bad signaling may participate in the regulation of excess tissue iron-induced caspase activation and cellular apoptosis and that deferasirox may act to diminish the degree of apoptosis, at least in part, by its ability to improve Akt signaling and the inactivation of Bad.

It is thought that iron-induced cardiac injury is initiated, at least partially, by the generation of reactive hydroxyl radicals, which, if excessive, can damage cellular macromolecules and lead to the initiation of cellular apoptosis (Ambrosio et al., 1987, 1998; Bolli et al., 1987). In vitro models that have examined the effects of elevated iron have shown that increased iron initially causes an elevation of Akt phosphorylation; however, this effect appears to be reversed after prolonged exposure (Kuperstein and Yavin, 2003; Chen et al., 2007). Why this shift from an antiapoptotic state (i.e., increased Akt phosphorylation) to one that is more permissive to apoptosis (i.e., diminished Akt phosphorylation) occurs is not well understood but may be related to long-term elevations in the amount of iron-associated reactive oxygen species (ROS). Supporting this contention, our recent data have demonstrated that persistent elevations in oxidative stress, such as that seen during the aging process, are associated with the post-translational modification (S-nitrosylation) of Akt and impairments in Akt enzymatic function and its downstream effectors (Wu et al., 2009b, 2010a,b, 2011). In the current study, we observe reduced Akt activation with iron overload and demonstrate that this finding is reversed by iron chelation. Whether combination-based therapies aimed at decreasing tissue iron levels and diminishing oxidative stress, perhaps through the concomitant administration of ROS scavengers, may lead to additional beneficial effects will require further study. However, it is interesting to note that the administration of deferoxamine combined with acetaminophen (a potent antioxidant) has demonstrated a greater capacity to remove cardiac iron in the iron-overloaded gerbil (Walker et al., 2009).

In summary, our data demonstrate that deferasirox treatment is associated with reduced cardiac fibrosis after iron overload. This effect is accompanied by a reduction of apoptosis, a finding that is related to a reduced cleavage of α-fodrin, improvements in cell membrane integrity, and decreased nucleic DNA fragmentation. These alterations appear to arise from deferasirox-associated decreases in caspase-3 activation that may be mediated by reduction in the proapoptotic Bax/Bcl-2 ratio and increased phosphorylation (inhibition) of Bad via Akt activation (Fig. 6). The findings of this study provide insight into the potential use of defera-
siox treatment for attenuating iron-induced cardiac tissue damage.

Authorship Contributions
Participated in research design: Wu and Blough.
Performed data analysis: Wu and Wang.
Wrote or contributed to the writing of the manuscript: Wu, Wang, and Blough.

Other: Blough acquired funding for the research.

References