Methylene blue rescues heart defects in a Drosophila model of Friedreich’s ataxia

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Friedreich’s ataxia (FRDA), the most common hereditary ataxia, is characterized by progressive degeneration of the central and peripheral nervous system, hypertrophic cardiomyopathy and a high risk of diabetes. FRDA is caused by abnormally low levels of frataxin, a highly conserved mitochondrial protein. Drosophila has been previously successfully used to model FRDA in various cell types, including neurons and glial cells. Here, we report the development of a Drosophila cardiac model of FRDA. In vivo heart imaging revealed profound impairments in heart function in frataxin-depleted Drosophila, including a strong increase in end-systolic and end-diastolic diameters and a decrease in fractional shortening (FS). These features, reminiscent of pathological phenotypes in humans, are fully rescued by complementation with human frataxin, suggesting conserved cardiac functions of frataxin between the two organisms. Oxidative stress is not a major factor of heart impairment in frataxin-depleted flies, suggesting the involvement of other pathological mechanisms notably mitochondrial respiratory chain (MRC) dysfunction. Accordingly, we report that methylene blue (MB), a compound known to act as an alternative electron carrier that bypasses mitochondrial complexes I-III, was able to prevent heart dysfunction. MB also partially rescued the phenotype when administered post-symptomatically. Analysis of MB derivatives demonstrates that only compounds with electron carrier properties are able to prevent the heart phenotype. Thus MB, a compound already used for several clinical applications, appears promising for the treatment of the heart dysfunctions that are a major cause of death of FRDA patients. This work provides the grounds for further evaluation of MB action in mammals.

INTRODUCTION

Friedreich’s ataxia (FRDA) is the most frequent autosomal recessive spinocerebellar ataxia among Caucasians with an incidence of ~1/50 000. It is a fatal disease characterized by progressive ataxia and dysarthria, and other non-neurological impairments that lead to death, at a mean age of ~37 years (1–4). The neurological symptoms, which include sensory neuropathy and deep sensory impairment, are linked to degeneration of the sensory neurons of the dorsal root ganglia, the corticospinal and spinocerebellar tracts of the spinal cord and the dentate nucleus. Other features of the disease are muscle weakness, diabetes and cardiac impairment. This last feature is the major non-neurological manifestation and was described by Friedreich in 1863 (5). The cardiomyopathy can already be observed in children, and patients with an earlier onset of disease generally also showed more severe cardiac involvement (6). The phenotype is mainly characterized by left ventricular hypertrophy, which may develop into dilated cardiomyopathy and progressive systolic dysfunction (1,6–14). Cellular and histological investigations have identified cardiomyocyte hypertrophy, focal necrosis and diffuse fibrosis in patients (14,15). Importantly, cardiac failure is the most common cause of death in FRDA (4).

The identification 16 years ago of the genetic defects associated with FRDA shed new light on the observed pleiotropic phenotypes. In almost all patients (96%), FRDA is caused by a GAA trinucleotide repeat expansion in the first intron of the gene encoding frataxin (FXN), an evolutionarily conserved mitochondrial protein (16). The repeat expansion involves 70–1700 repeats and impairs FXN transcription through various mechanisms [reviewed in (17,18)] causing partial...
depletion of the frataxin protein in the mitochondrial matrix (16). This results in four abnormalities, which may be linked together in a vicious circle: impairment of iron-sulfur cluster (ISC) synthesis or stability, decrease of aconitase and MRC activity (19), hypersensitivity to oxidative stress (20) and accumulation of iron inside mitochondria associated with depletion of cytosolic iron in affected organs (21–23).

Over the last ten years, several animal models have been developed to study the physiopathology of FRDA, including mouse knock-out (KO), knock-in and YAC models [(24–26), reviewed in (27)]. Neurological, cardiac and diabetic FRDA models have also been established (23,28–30), using conditional KO strategies that have allowed several tissue-specific features of the disease to be investigated. Interestingly, in the neuronal and cardiac loss-of-function models, pathological features may be observed in the absence of increased oxidative stress (31). These various models have thus facilitated fundamental investigations on the disease leading to the identification and detailed description of several affected pathways (22,32,33). They are also valuable tools for evaluating therapeutic interventions. Compounds that directly or indirectly prevent oxidative insults, such as idebenone (34) and PPARgamma agonists (35), or increase frataxin levels, such as the histone deacetylase inhibitor C106, have shown limited success in preventing the disease. Significant progress is still required to combat this disease.

Genetically tractable invertebrate models have emerged as an attractive alternative approach to mammalian models: they can be used for more rapid in vivo studies of animals presenting reduced frataxin levels in targeted tissues, to find genetic modifiers of the associated pathology and to screen for active compounds (36–42). Such models, in worms or flies, benefit from the short lifespan of these species and the strong evolutionary conservation of the frataxin protein and allow analyses of conserved pathological mechanisms and potential therapies.

We report an analysis of whether partial frataxin depletion in the fly heart impairs its function. We used a heart-specific inducible system to decrease the frataxin level, coupled to in vivo cardiac imaging in order to characterize the phenotype. Frataxin depletion was associated with substantial fly heart dilatation and impaired systolic function, mimicking the phenotypes observed in FRDA patients. Importantly, the impairments observed in frataxin-depleted Drosophila hearts are fully rescued by complementation with human frataxin, suggesting conserved functions of frataxin between the two organisms in this organ. Additional genetic and pharmacological approaches suggested that dysfunction in the MRC, but not oxidative stress, is a major factor of heart impairment in frataxin-depleted flies. Accordingly, we identified methylene blue (MB), a compound with mitochondrial electron transfer properties that provides protection in a rotenone-induced Parkinson model (43,44) and is already used for several clinical applications (45), as a potent therapeutic drug for FRDA heart impairment.

RESULTS
Frataxin depletion in the Drosophila heart leads to major cardiac dysfunction

To study the effect of heart-specific frataxin depletion, we down-regulated the expression of Drosophila frataxin homolog (fh) by RNA interference using a UAS-fhRNAi construct (42). The expression of fhRNAi was driven by the heart-specific RU486-inducible Geneswitch driver HandGS (46). The activity of the HandGS driver (and hence the level of frataxin depletion) is controlled by RU486 added to the fly food. The driver was simultaneously used to express a mitochondrial GFP, providing sufficient fluorescence in cardiomyocytes for high-speed video recording through the cuticle of anesthetized flies (Fig. 1A). Fluorescence labeling of the two heart edges enabled M-Mode analysis and quantification of several measures of cardiac function, including the heart period (HP), the end-systolic and diastolic diameters (ESD and EDD, respectively) and the FS, which provides an indication of the cardiac output. These analyses were conducted with automated tools that we have recently developed to study cardiac aging in Drosophila (46).

First, we confirmed that RU486 in the food did not affect heart performance using UAS-mitoGFP; HandGS> + (+) control flies (Supplementary Material, Fig. S1). Then, we fed UAS-mitoGFP; HandGS> fhRNAi flies with RU486 to study the effects of heart-specific frataxin depletion. We quantified the level of fh transcript in the heart of these flies by quantitative real-time PCR and observed that it was 60% lower than that in control flies (Fig. 1B).

fhRNAi flies showed striking cardiac defects (Fig. 1C and D, Supplementary Material, Videos S1 and S2). In these flies, ESD and EDD were 133 and 59% higher, respectively, than in control flies; FS was 49% lower and HP 28% higher than in control flies. We analyzed cardiac function at various ages and observed that the cardiac defects were already present in 2-day-old flies. These defects remained relatively stable during the first 2 weeks of age, with only a slight but statistically significant increase of EDD (Supplementary Material, Fig. S2). Frataxin inactivation starting from the third instar larva (by transferring larvae to RU486-containing medium at this stage) was also sufficient to cause heart dysfunction in young adults, whereas adult-specific frataxin inactivation did not lead to heart function defects, even at advanced ages (data not shown). These findings suggest that the fly heart is particularly sensitive to frataxin depletion during specific stages before adulthood.

Cardiac dysfunction in frataxin-depleted flies is rescued by human frataxin expression

Our analysis demonstrates that heart-specific depletion of frataxin in flies leads to increased ESD and EDD and reduced FS, indicating cardiac dilatation and impaired systolic function, respectively. These functional alterations are also observed in FRDA patients and in a mouse model of FRDA (6,23,34). In particular, mice in which the frataxin gene is completely deleted in striated muscles (conditional KO model based on the Cre-lox system) present a hypertrophic cardiomyopathy that evolves into a dilated cardiomyopathy.

To ensure that the heart phenotypes in Drosophila and mammals are related, we investigated whether human frataxin may rescue the phenotypes caused by the inactivation of endogenous Drosophila frataxin. We generated a UAS-hFXN transgenic strain in order to express the human frataxin (hFXN) in hearts depleted for the fly frataxin. The hFXN expression fully rescued the observed heart defects (Fig. 1B and C). All the functional heart parameters that were impaired in UAS-mitoGFP;
Figure 1. RNAi-mediated frataxin depletion leads to major heart dysfunctions. (A) To generate a cardiac model of FRDA, the Geneswitch RU486-inducible driver, specifically expressed in the heart under the control of the hand promoter (HandGS driver), was used to drive expression of GFP targeted to mitochondria (mitoGFP), thereby labeling the heart, and to mediate ds-RNA-mediated inactivation of the Drosophila fh gene. (B) qRT-PCR analysis of fh mRNA levels in hearts of 5-day-old w/ Y; UAS-mitoGFP/+; HandGS/+ (+) and w/Y; UAS-mitoGFP/UAS-fhRNAi; HandGS/+ (fhRNAi) male flies. The RpL32 gene was used as a reference. (C) Anterior part of hearts (abdominal segments A1/A2) observed through the cuticle under UV light in 10-day-old + and fhRNAi male flies. Representative M-Modes (generated by horizontal alignment of rows extracted at the same position for each movie frame) are shown. (D) End-systolic diameter (ESD, µm), end-diastolic diameter (EDD, µm), fractional shortening (FS, %) and heart period (HP, ms) in 10-day-old + (n = 21), fhRNAi (n = 42), hFXN (w/Y; UAS-mitoGFP/UAS-hFXN; HandGS/+; n = 27) and fhRNAi+hFXN (w/Y; UAS-mitoGFP/ UAS-fhRNAi, UAS-hFXN; HandGS/+, n = 25) male flies. All flies were fed with RU486 during development (20 ng/ml of food) and adulthood (100 µg/ml). All values are means (± SEM). Significant differences are indicated as follows: *P < 5 × 10^{-2}, **P < 5 × 10^{-3}.
Catalase is an antioxidant enzyme that detoxifies H₂O₂. Its over-dysfunction in frataxin-depleted hearts is responsible for the heart phenotype. Consequently, we tested whether some of these pathways or components of the mitochondrial respiratory chain, but not aconitase, induce heart dilatation in flies. This confirms that our Drosophila model is relevant to study the cardiac aspects of the FRDA disease and pertinent for genetic and pharmacological screening.

Increased antioxidant defense fails to prevent cardiac dysfunction in frataxin-depleted hearts

Catalase is an antioxidant enzyme that detoxifies H₂O₂. Its over-expression in flies depleted for frataxin in the peripheral nervous system (PNS) and oenocytes enhances resistance to H₂O₂ and restores lifespan and mitochondrial aconitase activity (39). This suggests that H₂O₂ is an important pathogenic substrate in these tissues. To determine whether similar mechanisms are involved in the heart, we overexpressed catalase in cardiomyocytes of flies depleted for frataxin. Catalase overexpression failed to reduce the heart dilatation and systolic dysfunction (Supplementary Material, Fig. S3A). We have also treated fhRNAi flies with EUK8, a synthetic superoxide dismutase and catalase mimetic that has been used successfully in flies to improve aging heart function (46). EUK8 treatment did not improve heart function in frataxin-depleted flies (Supplementary Material, Fig. S3B). This indicates that H₂O₂-induced oxidative stress is not a major contributor to the dilated heart phenotype induced by frataxin depletion in flies.

Inactivation of components of the mitochondrial respiratory chain, but not aconitase, induces heart dilatation in flies

Previous studies performed on heart tissue of FRDA patients and a mouse cardiac model of FRDA demonstrated reduced activities of the ISC-containing aconitase enzymes and MRC complexes I, II and III (19,23,47). We investigated whether such impairments are sufficient to induce heart dilatation in flies. First, we inactivated aconitase specifically in the heart, using a RNAi previously shown to effectively knock down the Acon gene when ubiquitously expressed (48): the functional heart phenotype induced by frataxin depletion in flies. This suggests that H₂O₂ is an important pathogenic substrate involved in the heart, we overexpressed catalase in cardiomyocytes of flies depleted for frataxin. Catalase overexpression failed to reduce the heart dilatation and systolic dysfunction (Supplementary Material, Fig. S3A). We have also treated fhRNAi flies with EUK8, a synthetic superoxide dismutase and catalase mimetic that has been used successfully in flies to improve aging heart function (46). EUK8 treatment did not improve heart function in frataxin-depleted flies (Supplementary Material, Fig. S3B). This indicates that H₂O₂-induced oxidative stress is not a major contributor to the dilated heart phenotype induced by frataxin depletion in flies.

Figure 2. MRC complex I and III deficiencies cause a dilated cardiomyopathy phenotype in flies. (A) Representative M-Modes of 10-day-old w/Y; UAS-mitoGFP/+; HandGS+ (+, control flies), w/Y; UAS-mitoGFP/UAS-fhRNAi; HandGS+ (+, fhRNAi flies), w/Y; UAS-mitoGFP/Acon-RNAi; HandGS+ (+, Acon-RNAi); w/Y; UAS-mitoGFP/CG1970-RNAi; HandGS+ (+, CG1970-RNAi); w/Y; UAS-mitoGFP/CG9172-RNAi; HandGS+ (+, CG9172-RNAi); w/Y; UAS-mitoGFP/CG12400-RNAi; HandGS+ (+, CG12400-RNAi); w/Y; UAS-mitoGFP/CG4769-RNAi; HandGS+ (+, CG4769-RNAi); and w/Y; UAS-mitoGFP/CG17856-RNAi; HandGS+ (+, CG17856-RNAi) female flies. CG1970, CG9172 and CG12400 are genes encoding components of MRC Complex I (CI); CG4769 and CG17856 are genes encoding components of MRC Complex III (CIII). (B) End-systolic diameter (ESD, μm) and end-diastolic diameter (EDD, μm) of 10-day-old flies treated or not with 30 μM MB (+ control flies (n = 13), fhRNAi (n = 8), Acon-RNAi (n = 17), CG1970-RNAi (n = 6), CG1970-RNAi + MB (n = 4), CG9172-RNAi (n = 18), CG9172-RNAi + MB (n = 27), CG12400-RNAi (n = 12), CG12400-RNAi + MB (n = 11), CG4769-RNAi (n = 29), CG4769-RNAi + MB (n = 30), CG17856-RNAi (n = 33), CG17856-RNAi + MB (n = 43). All flies were fed with RU486 during development (20 ng/ml of food, except Acon-RNAi and CG12400-RNAi flies fed with 100 ng/ml of food) and adulthood (100 μg/ml). All values are means (± SEM). Significant differences between flies of the same genotype treated or not with MB are indicated as follows: * P < 5 × 10⁻², ** P < 5 × 10⁻³.
(CG17856-RNAi flies), ESD and EDD were by 113 and 65% higher, respectively, than those in control flies.

These results suggest that respiratory chain deficiency could be a major mechanism involved in the heart dilatation owing to frataxin depletion. This hypothesis prompted us to test drugs known to improve electron transfer in the respiratory chain for their ability to prevent heart dilatation owing to respiratory chain and frataxin deficiencies.

**Treatment with idebenone failed to prevent heart dysfunction in frataxin-depleted hearts**

We examined the ability of idebenone, a compound proposed as a possible treatment for FRDA, to improve heart function in our cardiac model of FRDA. Idebenone is a short-chain synthetic analog of coenzyme Q10. Besides its antioxidant properties associated with inhibition of lipid peroxidation (49–52), idebenone also acts as an electron carrier in the mitochondrial electron transport chain, supporting mitochondrial function and ATP production. Several clinical studies have evaluated idebenone in FRDA patients. Reduction of cardiac hypertrophy was observed in several—but not all—studies (53,54) and idebenone delayed FRDA patients. Reduction of cardiac hypertrophy was observed in several—but not all—studies (53,54) and idebenone delayed FRDA patients. Reduction of cardiac hypertrophy was observed in several—but not all—studies (53,54) and idebenone delayed FRDA patients.

**Methylene blue treatment reduces heart dilatation induced by complexes I and III deficiencies**

Methylene blue cycles between oxidized and reduced states and functions as an alternative electron carrier in mitochondria (43,44,55). Therefore, MB was a good candidate to improve mitochondrial function in a context of respiratory chain deficiencies. We tested MB on flies with components of complex I or III with 30 µM MB. The heart dilatation was significantly decreased by the MB treatment in four of the five lines we have tested (Fig. 2B). A partial rescue was observed in CG12400-RNAi, CG4769-RNAi and CG17856-RNAi flies. In these latter, for example, ESD and EDD were 19 and 15% lower, respectively, than those in control flies. Strikingly, in CG9172-RNAi flies treated with MB, ESD and EDD were similar to those in control flies (P = 0.34 and P = 0.37, respectively) whereas in untreated flies, they were 59 and 27% higher than those in controls. We did not observe any significant effect of MB on CG1970-RNAi flies. However, the heart phenotype was very strong in these flies and ~80% of the hearts exhibited a very low GFP fluorescence, preventing analysis of cardiac function. This suggests that MB is not able to prevent heart dilatation in cases of strong complex I deficiency or that higher MB concentrations are required.

**Methylene blue treatment prevents heart dysfunction in frataxin-depleted hearts**

We hypothesized that the heart dilatation phenotype due to frataxin depletion was at least in part due to respiratory chain deficiency and could consequently be improved by MB treatment. We treated groups of fhRNAi flies with one of a series of amounts of MB (from 10 to 40 µM) during development: heart defects were decreased in a dose-dependent manner (Fig. 3 and Supplementary Material, Video S3). In fhRNAi flies treated with 30 µM MB, ESD, EDD and HP were similar to those observed in control flies (P = 0.23, P = 0.13 and P = 0.17, respectively) and FS was only slightly lower. Similar effects were observed with fhRNAi flies treated continuously during development and adulthood (data not shown). Frataxin depletion in these flies is dependent on RU486 feeding and Geneswitch activity. Thus, we checked that MB did not affect this inducible system, to exclude artificial effects. We assayed GFP protein in HandGS;UAS-mitoGFP > + flies treated or not treated with MB. GFP expression was not affected by MB treatment (Supplementary Material, Fig. S5A). The extent of frataxin depletion in daGS > UAS-fhRNAi flies, in which expression of fhRNAi was driven ubiquitously by the daGS RU486-inducible Geneswitch driver, was also quantified: the fh transcript was 55% lower than that in daGS > + control flies, and this level of depletion was not affected by MB treatment (Supplementary Material, Fig. S5B). These results indicate that MB did not affect larval feeding or Geneswitch mediated inactivation of frataxin and confirm that the rescue of heart dysfunction by MB treatment is not an artifact.

**Post-symptomatic treatment with methylene blue partially rescues heart dysfunction in frataxin-depleted hearts**

We studied the effects of post-symptomatic MB treatment. We treated fhRNAi flies at the adult stage (from the age of 2 days), when the cardiac defects were already established, and performed cardiac imaging at the age of 15 days. Cardiac variables improved significantly under MB treatment, and in a highly dose-dependent manner (Fig. 4). The largest effects were observed with the 15 µM MB treatment: ESD and EDD were 43 and 19% higher, respectively, than those in control flies, whereas in untreated fhRNAi flies, they were 107 and 51% higher. Similarly, FS was 35% lower in untreated fhRNAi than that in controls and was only by 17% lower than control values following 15 µM MB treatment, and HP was restored to the wild-type level. However, 5 and 30 µM MB treatment failed to improve heart function, suggesting that the therapeutic window, at least in the case of post-symptomatic treatment, is limited to a small range.

**Effects of MB derivatives on the heart dilatation induced by frataxin deficiency**

Next, we investigated on our in vivo cardiac model the structure–activity relationships of MB, in regards to a previous in vitro study using MB and structurally related compounds (56).
We tested five pharmacologically MB derivatives for their ability to prevent heart dilatation in fhRNAi flies. These derivatives were: toluidine blue (TB, with amine side chains attached at positions 3 and 7 of the phenothiazine nucleus, similar to MB), Neutral Red (NR, substitution of sulfur at position 5 with nitrogen), 2-chlorophenothiazine (2CP, side chain deletions at positions 3 and 7) and Chlorpromazine and Promethazine (CP and P, side chain deletions at positions 3 and 7 and substitution of a
side chain at position 10). TB was the only MB derivative with beneficial effects on heart dilatation following 30 mM treatment (Fig. 5). However, it was less efficient than MB: the 10 mM MB treatment failed to reduce dilatation, when the 10 mM MB treatment had significant effects.

Interestingly, MB and TB both act as alternative electron carriers that bypass mitochondrial complexes I–III, whereas the other derivatives do not (56). So, we observed a good correlation between the mitochondrial electron transfer activity and the ability of compounds to prevent heart dilatation, suggesting that this property is involved in the cardioprotective effect.

**DISCUSSION**

The fruit fly *Drosophila melanogaster* has recently emerged as a useful model to study cardiac diseases. Although the Drosophila heart is structurally different from the complex chambered mammalian heart, it must be emphasized that Drosophila cardiomyocytes share many hallmarks of mammalian cardiac cells: they are mononucleated striated muscle cells with sarcomeric apparatus very similar to the vertebrate ones, and many proteins involved in cardiac function, such as ion channels and contractile proteins, are highly conserved between flies and humans (57, 58). The fly heart combines advantages of invertebrate models (the availability of genetic tools, studies on large populations) with suitability for physiological studies thanks to several recently developed cardiac imaging methods (59–61). We generated a new Drosophila model of FRDA, in which the Drosophila frataxin homolog was inactivated specifically in cardiomyocytes. This Drosophila model recapitulates the defects of cardiac function observed in patients and mouse models of FRDA, and in particular heart dilatation and impaired systolic function. Importantly, the fly heart phenotype is fully rescued by expression of human frataxin in cardiomyocytes. This result validates our model at two levels: first it shows that the cardiac defects are due to frataxin depletion and excludes off-target effects of the RNAi construct, and second it demonstrates that fly and human frataxins share similar functions in cardiomyocytes. This Drosophila cardiac model of FRDA is also a powerful model for pharmacological screening because it allows to test a set of compounds on a large number of flies in a relatively short time (50 flies can be recorded in one hour, and each step of the analysis is automated), as illustrated here in a pilot study on MB derivatives.

Using this model, we investigated the mechanisms involved in heart dysfunctions in frataxin-depleted animals by genetic and pharmacological approaches with emphasis on oxidative stress...
and mitochondrial dysfunction. Oxidative stress has been suggested to be involved in the pathophysiology of FRDA. In particular, elevated levels of oxidative stress markers have been observed in patient samples (62–64) and cultured fibroblasts from patients exhibit increased sensitivity to oxidative stress (65). Accordingly, overexpression of catalase, an H₂O₂-scavenging enzyme, suppresses the deleterious phenotype associated with frataxin depletion in the fly PNS and oenocytes (39). However, catalase overexpression failed to improve cardiac function in frataxin-depleted flies. This result was confirmed pharmacologically with the SOD/catalase mimic EUK8. In vivo treatment with this compound prevents age-related cardiac impairments in wild-type flies (46) and improves heart function in pathological contexts in mammals (66,67). In contrast, we found that EUK8 failed to prevent heart defects associated with frataxin depletion in Drosophila. Similarly, in a

Figure 5. Compared activities of MB and derivatives on the heart dilated phenotype induced by frataxin depletion. (A) Chemical structure of MB and derivatives. (B) End-systolic diameter (ESD, µm) and end-diastolic diameter (EDD, µm) of 10-day-old w/T: UAS-mitoGFP/+; HandGS/+ control (n = 21) and w/T: UAS-mitoGFP/UAS-fhRNAi; HandGS/+ male flies untreated (n = 23) or treated during development with MB (10 µM n = 22, 30 µM n = 17), Toluidine Blue (10 µM n = 12, 30 µM n = 19), Neutral Red (10 µM n = 18, 30 µM n = 15), 2-chlorophenothiazine (10 µM n = 18, 30 µM n = 15), Promethazine (10 µM n = 25, 30 µM n = 13) and Chlorpromazine (10 µM n = 15, 30 µM n = 14). All flies were fed with RU486 during development (20 ng/ml of food) and adulthood (100 µg/ml). All values are means (±SEM). Significant differences with values for fhRNAi flies not treated with MB or derivatives are indicated as follows: ∗P < 5 × 10⁻³.
mouse model for FRDA, the MnSOD mimetic (MnTBAP) has no beneficial effect on cardiomyopathy (31). Altogether, this suggests that, at least in flies and mice, oxidative stress is not a major contributor to the heart phenotypes observed in FRDA.

Then, several observations prompted us to investigate whether MRC deficiencies could be responsible for cardiomyopathies in Frataxin-depleted flies. A deficient activity of MRC complexes I, II and III was reported in the endomyocardial biopsy of two unrelated FRDA patients (19). A second study performed on heart tissues of ten FRDA patients also described significant decreases in complex I and complex II/III activities (47). Mice in which the frataxin gene is fully deleted in striated muscles (MCK mouse, conditional KO model based on the Cre-lox system) present a hypertrophic cardiomyopathy, which evolves into a dilated cardiomyopathy, associated with reduced complex I, II and III activities (23,34). In flies, ubiquitous frataxin depletion leads to reduced activities of complexes I, II, III and IV by 40–60% (42). In addition, defective MRC enzyme activities have been extensively reported to be associated with dilated and hypertrophic cardiomyopathy in humans [reviewed in (68,69)], suggesting a high sensitivity of the heart to MRC perturbations. To investigate whether MRC deficiencies could be responsible for the dilated phenotype in frataxin-depleted hearts, we have inactivated several component of complex III and complex I in flies, including orthologs of NDUFS2 and NDUFS7, two genes associated with human diseases: NDUFS7 mutations were associated with Leigh syndrome in several families (70–72) and mutations in the NDUFS2 gene have been found in patients with Leigh syndrome in some cases associated to hypertrophic cardiomyopathy (73). Interestingly, in the fly model, we observed a significant heart dilatation following inactivation of the five genes encoding MRC complex I and III components. Altogether, these data strongly suggest, although by indirect evidence, that MRC dysfunction is a major pathophysiological mechanism involved in cardiac dysfunction in FRDA. As frataxin deficiency results in pleiotropic effects, other pathophysiological mechanisms could also be involved. Although reduced aconitase activity and iron accumulation have been observed in the heart tissue of FRDA patients (19,47), we found that aconitase inactivation did not induce heart dilatation and is consequently not involved in the cardiac phenotype owing to frataxin depletion in flies.

Cardiac iron metabolism is strongly affected in the MCK mouse, with mitochondrial iron-loading associated with cytosolic iron deficiency (22,23,34,74,75). It would be informative to determine whether similar iron metabolism defects also occur in Drosophila cardiomyocytes depleted for frataxin, although the small heart size and the limited number of cells within a single fly heart limit biochemical approaches.

Next, we examined the ability of two compounds known to potentiate mitochondrial function, idebenone and MB, to improve heart function in frataxin-depleted hearts. We did not observe any significant improvement of heart function with idebenone treatment. However, we cannot exclude the possibility that these negative results are due to inappropriate doses, insufficient absorption or distribution or rapid degradation of this drug in flies.

In contrast to idebenone, MB treatment strongly improves the function of frataxin-depleted hearts. MB is an autoxidizable heterocyclic aromatic dye, has a very low redox potential and cycles very efficiently between oxidized (MB) and reduced (MBH2, leuko MB) forms. MB is a direct substrate of NADH dehydrogenase in the MRC complex I, which converts MB into MBH2 (44). MBH2 can donate electrons to coenzyme Q and cytochrome c, and consequently increase cytochrome c oxidase activity (complex IV) and oxygen consumption (44,76,77). This property enables MB to bypass complex I/III blockage. Indeed, MB prevents striatal neurodegeneration, and neurological and behavioral deficits in rats following intrastratal infusion with rotenone, an inhibitor of complex I (43,44). In agreement, we observed that MB significantly improved heart dilation in flies depleted for several components of MRC complexes I and III.

MB effectively prevented heart dysfunction owing to frataxin depletion. When administered during fly development, we observed a full rescue of all cardiac functions (ESD, EDD, FS and HP) in the frataxin-depletion conditions tested. When MB was administered post-symmetrically in adults, we observed partial rescue of heart defects. This shows that MB can be protective post-symmetrically but also highlights the importance of an early administration to maximize its beneficial effect. Moreover, the response was highly dose-dependent when MB was administered to adults, the higher concentrations being less effective. Dose-dependent responses to MB have been reported in several cases, with low and high doses having opposite effects (55). For instance, MB administered to rat brain homogenates increased cytochrome c oxidase activity at low doses but reduced this activity at high doses (77). This highlights the importance of determining and using appropriate doses of MB if it is to be administered to humans. Our functional analysis of MB derivatives suggests that the beneficial effect of MB is linked to its alternative electron carrier ability, shared by toluidine blue. However, according to the pleiotropic properties of MB, notably its effect on heme synthesis and its antioxidant properties illustrated by a recent report showing protective effect of MB against BSO-induced toxicity in FRDA fibroblasts (78), we cannot exclude that other mechanisms may participate to the rescue of heart dysfunctions in FRDA.

MB is used in humans for several indications including methemoglobinemia, septic shock and ifosfamide-induced encephalopathy (45,79–82). MB is well tolerated by humans, crosses the blood–brain barrier and has recently been proposed as a neuroprotective compound, according to its alternative mitochondrial carrier, antioxidant and antiaggregative properties. MB has been tested in several vertebrate models of degenerative diseases with contrasting results. MB has shown some protective effects in amyotrophic lateral sclerosis models with expression of FUS, TDP-43 (83) and SOD1 (84) mutant proteins. In Alzheimer’s disease and related models, MB showed limited protective effects in 3x-Tg-AD mice (85) and reduced Tau levels by inducing autophagy (86) although improvements of behavior or lifespan have not been reported in this latter study. In contrast, MB failed to inhibit Tau and polyglutamine protein-dependent toxicity in zebrafish models (87). The strongest protective effects of MB have been observed with models of toxicity induced by inhibitors of MRC enzymes (44,88,89). Notably, MB appears to be highly effective in a sporadic Parkinson disease model with rotenone treatment (44).

Our results suggest that MB is a promising candidate for the treatment of FRDA. We expect that these findings stimulate
further work to assess its efficacy in mammalian models of FRDA and rapidly lead to clinical applications.

**MATERIALS AND METHODS**

**Drosophila stocks and culture methods**

USAS-CatIR2Ai (w[1] Pw[+mC] = USAS-CatIR2), USAS-Cat [w[1]; Pw[+mC] = USAS-Cat.A2] and USAS-mitoGFP [w[1118]; Pw[+mC] = USAS-mitoGFP.AP2CyO] were obtained from the Bloomington Stock Center. HandGS and daGSGeneswicht drivers are described in Monnier et al. (46) and Tricoire et al. (90). CG1970-RNAi (w[1118]; PGD6427-v38224), CG9172-RNAi (w[1118]; PGD6427-v38224), CG9172- Pw[5]; PGD3652-v37462/TM3), CG4769-RNAi (w[1118]; PGD3652-v37462/TM3), CG4769-RNAi (w[1118]; PGD3851-v9180), CG17856-RNAi (w[1118]; PGD9481-v33015) and Acon-RNAi (w[1118]; PGD1348-v12455) were obtained from the VDRC Stock Center.

For RU486 induction during development, eggs were allowed to develop on fly medium supplemented with RU486 (from a 20 mg/ml stock solution in ethanol) as described in Tricoire et al. (90) and Rera et al. (91). Adult flies were collected within 24 h of eclosion under brief CO2 anesthesia and housed in groups of 20. Flies were raised at 26°C under a 12–12 h light cycle and transferred every 2 days onto fresh food.

**Treatment with compounds**

Compounds were incorporated in the food medium. Stock solutions (10 mM in deionized water, unless otherwise mentioned) of EUK8 (CALBIOCHEM), idebenone (SIGMA, 25 mg/ml in ethanol), Methylene Blue (SIGMA, 5 mM in deionized water), Toluidine Blue (SIGMA), Neutral Red (RAD diagnostics), 2-chlorophenothiazine (SIGMA, 10 mM in ethanol), Chlorpromazine (SIGMA) and Promethazine (SIGMA) were prepared and stored at 4°C.

**In vivo imaging of fly hearts**

Flies expressing the GFP protein targeted to the mitochondria (mitoGFP) to label the cardiac tube were anesthetized with triethylamine (FlyNAP) and observed under a Zeiss SteREO Lumar.V12 Stereomicroscope, with a NeoLumar S 1.5 objective. Videos were acquired with an AxioCamHR camera. M-Modes were generated by horizontal alignment of rows extracted at the same position from each movie frame by using ImageJ. Cardiograms (defined by the distance between the maxima of GFP fluorescence on each side of the median position of the heart at each time point) were then generated from M-Modes using an image processing algorithm developed with Matlab R2010b. The temporal positions for each end-systolic and end-diastolic position of the heart were extracted by finding all local maxima and minima on the cardiogram. The resulting file was incorporated into an Access Database to extract, for each cardiogram, the HP and the Arrhythmicity Index. Statistical significance was assessed by non-parametric Wilcoxon analysis.

Details of the M&M can be found in the supporting information.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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