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Highly specific ubiquitin-competing molecules effectively promote frataxin accumulation and partially rescue the aconitase defect in Friedreich ataxia cells

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ABSTRACT

Friedreich ataxia is an inherited neurodegenerative disease that leads to progressive disability. There is currently no effective treatment and patients die prematurely. The underlying genetic defect leads to reduced expression of the mitochondrial protein frataxin. Frataxin insufficiency causes mitochondrial dysfunction and ultimately cell death, particularly in peripheral sensory ganglia. There is an inverse correlation between the amount of residual frataxin and the severity of disease progression; therefore, therapeutic approaches aiming at increasing frataxin levels are expected to improve patients' conditions. We previously discovered that a significant amount of frataxin precursor is degraded by the ubiquitin/proteasome system before its functional mitochondrial maturation. We also provided evidence for the therapeutic potential of small molecules that increase frataxin levels by docking on the frataxin ubiquitination site, thus preventing frataxin ubiquitination and degradation. We called these compounds ubiquitin-competing molecules (UCM). By extending our search for effective UCM, we identified a set of new and more potent compounds that more efficiently promote frataxin accumulation. Here we show that these compounds directly interact with frataxin and prevent its ubiquitination. Interestingly, these UCM are not effective on the ubiquitin-resistant frataxin mutant, indicating their specific action on preventing frataxin ubiquitination. Most importantly, these compounds are able to promote frataxin accumulation and aconitase rescue in cells derived from patients, strongly supporting their therapeutic potential.

Introduction

Friedreich ataxia (FRDA) is a genetic neurodegenerative disease that affects children and young adults and leads to progressive disability and premature death (Pandolfo and Pastore, 2009). It has an autosomal recessive inheritance with an estimated prevalence of 1:50,000 in the Caucasian population, thus representing the most common form of inherited ataxia. Characteristic symptoms include progressive loss of movement coordination, gait instability, muscle weakness and sensory loss. Symptoms usually appear around puberty and patients usually require wheelchairs within 10 to 15 years from disease onset. Disease progression is often associated with loss of visual acuity, slurred speech and dysphagia. Neurological signs are associated with degeneration of sensory neurons in the dorsal root ganglia and dentate nucleus of the cerebellum. Moreover, patients often develop a hypertrophic cardiomyopathy that is often the cause of premature death (Weidemann et al., 2013). A significantly higher incidence of diabetes mellitus is also associated with the disease, with more than 25% of patients developing glucose intolerance or diabetes (Cnop et al., 2013). The disease is caused by a GAA triplet-repeat expansion within the first intron of the gene coding for frataxin (Campuzano et al., 1996), which results in reduced transcription of the gene. The vast majority of patients present a homozygous repeat expansion on both alleles, while about 4% of patients present a GAA repeat expansion on one allele and a point mutation in the coding region on the other allele. In normal individuals the number of GAA triplets range between 10 and 35, while in affected individuals GAA triplets range from 66 up to more than 1700 (Yandim et al., 2013). This is associated with a number of epigenetic changes that lead to heterochromatinization of this portion of DNA and impairment

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of gene transcription (Al-Mahdawi et al., 2008; Greene et al., 2007). The formation of an atypical "sticky" triple DNA structure has also been found associated with the expanded GAA triplet, also accounting for the observed gene silencing (Bidichandani et al., 1998; Sakamoto et al., 2001). The genetic defect results in a severely reduced transcription of the frataxin gene, with patients living with residual 10-30% frataxin (Yandim et al., 2013).

Frataxin is a highly conserved mitochondrial protein (Pastore and Puccio, 2013), synthesized in the cytosol as a precursor form, which is then processed by a mitochondrial processing peptidase in a two-step catalytic process, and concomitantly imported into mitochondria (Koutnikova et al., 1998). The functional mature form is a 130 amino acid polypeptide (Condò et al., 2007), mainly located within the mitochondrial matrix. Frataxin is involved in iron metabolism and participates in the biogenesis of iron-sulfur clusters (ISC) (Vaubel and Isaya, 2013). It is therefore essential for the enzymatic activity of complex I, II and III of the mitochondrial respiratory chain and of aconitases (Bulteau et al., 2004; Condò et al., 2010; Rotig et al., 1997), which require ISC as cofactors. Therefore, frataxin-deficient cells present an impairment in the electron transport chain and inefficient mitochondrial respiration. Iron distribution is consequently affected in frataxin-deficient cells, resulting in intramitochondrial iron overload (Martelli and Puccio, 2014). As a result of dysregulated mitochondrial metabolism, frataxin-deficient cells have reduced ATP content and increased ROS generation. Moreover, frataxin insufficiency results in impaired intracellular anti-oxidant defenses (Paupe et al., 2009; Shan et al., 2013).

To date, no effective therapy has been approved to treat FRDA. Frataxin insufficiency is considered the main pathogenic cause of the disease and a correlation exists between the length of the GAA expansion and the extent of transcription impairment. Moreover, the amount of residual frataxin has been correlated to the age at disease onset and to the severity of disease progression (Parkinson et al., 2013). Since the coding sequence of frataxin is unaltered in patients, therapeutic approaches aiming at increasing frataxin levels (Herman et al., 2006; Marmolino et al., 2009; Tomassini et al., 2012) are therefore expected to ameliorate disease symptoms and to slow down disease progression. Both promoting frataxin gene expression and preventing frataxin protein degradation can in principle lead to an increase in frataxin levels. We recently focused our studies on the therapeutic potential of preventing frataxin degradation to promote its accumulation.

We have previously shown that frataxin levels are controlled by the ubiquitin/proteasome system (UPS) and that frataxin can be directly modified by ubiquitin (Rufini et al., 2011). The UPS is the most important and widely studied system for intracellular protein degradation. Lysine residues on the protein substrate are recognized by specific enzymes and modified by the covalent attachment of one or more ubiquitin moieties. This event marks the protein for degradation in the proteasome (Glickman and Ciechanover, 2002). We identified the critical lysine on frataxin, lysine 147 (K147), which is the main target of ubiquitination. This lysine represents a crucial site for frataxin stability. Indeed a frataxin mutant that lacks this lysine cannot be ubiquitinated and is more stable. Therefore, preventing ubiquitination on K147 is expected to grant frataxin an increased stability and a prolonged half-life. Indeed, from computational docking studies, we identified a set of small molecules, predicted to interact with the molecular pocket surrounding K147, able to interfere with frataxin ubiquitination and promote frataxin accumulation in cells derived from patients. Moreover, these molecules, named ubiquitin-competing molecules (UCM), can promote a functional rescue of mitochondrial dysfunction caused by frataxin deficiency in patients cells (Rufini et al., 2011).

We have now extended our search to find more effective compounds. We show here that these "second-generation" UCM, physically interact with frataxin and prevent its ubiquitination. Moreover, these compounds can increase frataxin levels in cells overexpressing frataxin, but not in cells overexpressing the ubiquitin-resistant K147R frataxin mutant, suggesting that they act by inhibiting ubiquitination on K147. Importantly, they show efficacy in promoting accumulation of mature frataxin, and in restoring aconitase activity in cells derived from patients, strongly supporting their potential therapeutic application.

Results

Computational screening for ubiquitin-competing molecules (UCM)

In order to design small molecules able to inhibit the ubiquitination of frataxin, an extended analysis of the protein's accessible surfaces has been performed, extending our previous work, by taking into account protein flexibility. This analysis allowed us to identify binding pockets on frataxin that were more accessible to drugs. By focusing our analysis on the areas more proximal to K147, and by using virtual screening of commercially available compound libraries, several thousand compounds were docked on available NMR and x-ray structures of human frataxin. Some of these molecules were predicted to interact with frataxin near to K147 (Fig. 1). Promising candidates were subjected to functional validation.

UCM increase frataxin levels

To validate UCM activity, we tested their effect in human HEK-293 cells stably expressing single copy frataxin (293-frataxin). These cells allow the detection of all forms of frataxin, including the frataxin precursor. Compounds that were able to enhance frataxin precursor levels were further chemically modified to better fit the docking model, synthesized and tested again in 293-frataxin. This process was repeated in an iterative cycle with the aim to improve the efficacy of the UCM. Approximately 200 new candidate UCM were tested in functional assays. Through this process, we were able to identify new UCM that promote frataxin precursor accumulation more efficiently than the previously described compounds. Structures of the compounds described in this study are shown in Table 1. Indeed, the treatment of 293-frataxin cells with 10 μM UCM53, UCM108 and UCM71 is able to induce frataxin precursor accumulation (Fig. 2A) more efficiently than with the previously described UCM2 (referred to as NSC620301 in (Rufini et al., 2011)) or with the proteasome inihibitor MG132. Importantly, an accumulation of mature frataxin can also be observed in these cells when treatment is prolonged for 3 days (Fig. 2B).

UCM prevent frataxin ubiquitination

To test whether the new compounds promote frataxin accumulation by preventing its UPS-dependent degradation, we evaluated their impact on frataxin ubiquitination. To this aim, we performed an *in vivo* ubiquitination assay. HEK-293 cells were transiently co-transfected with hemagglutinin-tagged ubiquitin (HA-Ub) and frataxin, in the presence of proteasome inhibitor and deubiquitinase inhibitor to allow the accumulation of ubiquitinated species, in the presence of the selected compounds. The ubiquitination status of frataxin was evaluated by SDS–PAGE of total cell lysates and anti-frataxin immunoblotting. As previously described, in this experimental setting, frataxin monoubiquitinated forms can be detected by anti-frataxin antibody as a slower migrating band above frataxin precursor (Rufini et al., 2011). Ubiquitination level was measured as the ratio between the levels of ubiquitinated frataxin and frataxin precursor. As shown in Fig. 3, UCM53 and UCM71 but not the control non-effective molecule UCM57, can significantly abrogate frataxin ubiquitination. These data suggest that the selected UCM interfere with frataxin ubiquitination in living cells.

UCM promote frataxin accumulation by preventing K147-dependent degradation

We had previously shown that K147 is the crucial ubiquitination site on frataxin. Since we showed that these compounds are able to