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Small molecules affecting transcription in Friedreich ataxia

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Abstract

This review concerns the development of small molecule therapeutics for the inherited neurodegenerative disease Friedreich ataxia (FRDA). FRDA is caused by transcriptional repression of the nuclear *FXN* gene, encoding the essential mitochondrial protein frataxin, and accompanying loss of frataxin protein. Frataxin insufficiency leads to mitochondrial dysfunction and progressive neurodegeneration, along with scoliosis, diabetes and cardiomyopathy. Individuals with FRDA generally die in early adulthood from the associated heart disease, the most common cause of death in FRDA. While anti-oxidants and iron chelators have shown promise in ameliorating the symptoms of the disease, there is no effective therapy for FRDA that addresses the cause of the disease, the loss of frataxin protein. Gene therapy and protein replacement strategies for FRDA are promising approaches; however, current technology is not sufficiently advanced to envisage treatments for FRDA coming from these approaches in the near future. Since the *FXN* mutation in FRDA, expanded GAA·TTC triplets in an intron, does not alter the amino acid sequence of frataxin protein, gene reactivation would be of therapeutic benefit. Thus, a number of laboratories have focused on small molecule activators of *FXN* gene expression as potential therapeutics, and this review summarizes the current status of these efforts as well as the molecular basis for gene silencing in FRDA.

Keywords

frataxin; Friedreich ataxia; transcription; polyamides; histone deacetylase inhibitor

1. Introduction: Friedreich ataxia, the gene mutation and molecular basis for the disease

Friedreich ataxia (FRDA) is the most prevalent inherited ataxia (reviewed in (Pandolfo, 1999)), affecting approximately 1:50,000 individuals. FRDA is caused by deficiency of the protein frataxin, a highly conserved nuclear-encoded, mitochondrial protein. Frataxin is believed to be involved in iron homeostasis, storage and transfer of iron-sulfur clusters to partner proteins, and energy production in the cell, but its precise cellular function is controversial (Bencze et al., 2006; Bulteau et al., 2004; Seznec et al., 2005). Frataxin insufficiency leads to progressive spinocerebellar neurodegeneration, causing symptoms of gait and limb incoordination, slurred speech, muscle weakness, and loss of position and vibration sense, with extraneural scoliosis, cardiomyopathy, and diabetes. At present there is no effective treatment for FRDA, and generally within 15 to 20 years after the first appearance

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of symptoms, affected individuals are confined to a wheelchair. Individuals with FRDA generally die in early adulthood from the associated heart disease.

FRDA is an autosomal recessive disease in which the majority of patients (~98%) inherit an expanded region of GAA·TTC triplet-repeats in the first intron of the frataxin (*FXN*) gene from both parents (Campuzano et al., 1996). Typically, the parents of FRDA patients are themselves asymptomatic heterozygous carriers of this expansion in one *FXN* allele. A small number of patients have one expanded allele and a second allele harboring a premature stop codon or point mutation (Bidichandani et al., 1997). FRDA patients have a marked deficiency of frataxin mRNA (Bidichandani et al., 1998; Campuzano et al., 1996), providing evidence that the expanded repeats cause gene silencing. The extent of frataxin deficiency is related to the length of the GAA·TTC repeats (Pianese et al., 2004). Individuals who are heterozygous for the mutation also have reduced levels of frataxin, but are asymptomatic. Two models have been put forward to explain *FXN* gene silencing by triplet expansion: In the first model, transcription inhibition is believed to be caused by an unusual DNA structure adopted by the GAA·TTC repeats (see below), which interferes with transcriptional elongation. In the second model, the GAA·TTC repeats, which resemble transcriptionally silent satellite DNA, recruit heterochromatin binding proteins, and cause gene silencing through an inactive chromatin structure. Normal *FXN* alleles have 6 – 34 repeats while FRDA patient alleles have 66 – 1700 repeats. Longer repeats cause a more profound frataxin deficiency and are associated with earlier onset and increased severity of the disease. Yeast cells deficient in the frataxin homologue accumulate iron in mitochondria and show increased sensitivity to oxidative stress (Babcock et al., 1997). This suggests that Friedreich ataxia is caused by mitochondrial dysfunction and free radical toxicity, with consequent mitochondrial damage, axonal degeneration, and cell death. Human frataxin is likely involved in similar processes since it is a mitochondrial protein, and FRDA patients have abnormal myocardial iron deposits (Harding, 1993). Based on these findings, antioxidant and iron-chelation based strategies appear promising in counteracting the course of the disease (Boddaert et al., 2007; Hart et al., 2005; Richardson, 2003; Rotig et al., 2002; Seznec et al., 2004). However, these strategies only treat the symptoms of the disease and not its cause; thus, pursuit of other approaches that address the cause of the disease are worthwhile. In this regard, stem cell therapy (Galvin and Jones, 2006), protein replacement (Mackenzie and Payne, 2007), and gene therapy (Gomez-Sebastian et al., 2007) could certainly correct frataxin deficits in affected individuals; however, these technologies are not sufficiently advanced to expect success for a neurological disease in the near term. Thus, pharmacological reactivation of the silenced *FXN* gene has received considerable attention over the past few years.

Extensive biochemical studies have documented that expanded GAA·TTC repeats adopt an unusual DNA structure ((Bidichandani et al., 1998; Ohshima et al., 1998; Sakamoto et al., 1999), and additional references therein). Long GAA·TTC repeats form triplexes containing two purine (R) GAA strands along with one pyrimidine (Y) TTC strand, flanking a single-stranded pyrimidine region. Formation of the RRY triplex in plasmid DNA requires divalent metal ions and negative supercoiling. A high-resolution NMR structure of a GAA·TTC·GAA triplex has been presented (Mariappan et al., 1999). Additionally, RRY triplexes have been shown to adopt “sticky” DNA structures *in vitro* (Sakamoto et al., 1999). Sticky DNA is detected by reduced mobility of DNA restriction fragments containing GAA·TTC repeats on gel electrophoresis and likely arises from intramolecular association of long GAA·TTC repeat triplexes. An excellent cartoon visualization of a model for the formation of intramolecular sticky DNA is provided as supplementary on-line information to the paper by Sakamoto and colleagues (Sakamoto et al., 1999). Sticky DNA formation has been shown to inhibit transcription both *in vitro* and in transfection assays in mammalian cells (Sakamoto et al., 2001). Using cloned repeat sequences from FRDA patients, GAA·TTC repeats have been found to interfere with *in vitro* transcription in a length-dependent manner (Bidichandani et al.,

1998; Grabczyk and Usdin, 2000b; Ohshima et al., 1998). This interference is most pronounced in the physiological orientation of transcription (i.e., synthesis of the GAA-rich transcript). These results are consistent with the observed correlation between repeat length, triplex formation and the age at onset and severity of disease. GAA·TTC repeat-mediated inhibition of replication in mammalian cells also provides strong evidence for the formation of RRY triplexes and sticky DNA in cells (Krasilnikova et al., 2007).

In contrast to the DNA structure-based mechanism for gene silencing by long GAA·TTC repeats, a study employing artificial transgenes for the lymphoid cell surface marker protein hCD2 has shown that expanded GAA·TTC repeats induce repressive heterochromatin *in vivo*, in a manner reminiscent of position effect variegated gene silencing (PEV) (Saveliev et al., 2003). PEV occurs when a gene is located within or near regions of heterochromatin, and silent heterochromatin is characterized by the presence of particular types of histone modifications (for example, H3-K9 trimethylation and histone tail hypoacetylation), the presence of histone deacetylases, DNA methyltransferases, chromodomain proteins, such as members of the HP-1 family of repressors, and polycomb group proteins (Elgin and Grewal, 2003). To investigate the role of chromatin structure in GAA·TTC repeat-associated gene silencing, Festenstein and co-workers (Saveliev et al., 2003) found that the hCD2 transgene harboring GAA·TTC repeats is resistant to DNase I digestion at the promoter element, compared to the normal transgene lacking GAA·TTC repeats. An increase in nucleosome density within the GAA·TTC repeats themselves was also found. Additionally, GAA·TTC repeat-mediated repression was enhanced by co-expression of the heterochromatin protein HP1 (Saveliev et al., 2003). It is certainly possible that an unusual non-B DNA structure, such as the triplexes and/or the sticky DNA conformation adopted by GAA·TTC repeats, is the signaling mechanism that initiates silencing of genes harboring such repeats, such as the *FXN* gene in FRDA. Proteins that recognize and bind these alternative DNA structures might initiate a cascade of events, such as histone postsynthetic modification (deacetylation and/or methylation of specific amino acid residues) and recruitment of heterochromatin proteins, which ultimately lead to gene silencing.

Taken together, the results on GAA·TTC repeat DNA and chromatin structure lead to a hypothesis that molecules that interfere with triplex/sticky DNA formation and/or heterochromatin formation in the *FXN* gene would likely increase successful elongation through expanded GAA·TTC repeats, thereby relieving the deficiency in *FXN* mRNA and protein in affected individuals (Grabczyk and Usdin, 2000a; Napierala et al., 2004; Saveliev et al., 2003). Additionally, molecules that increase *FXN* promoter activity, either acting on DNA or the protein constituents of the transcription initiation or RNA chain elongation complexes, could be therapeutic for FRDA (Sarsero et al., 2003). This review will focus on the identification and characterization of such molecules. Recent excellent reviews have focused on the etiology of FRDA (Lodi et al., 2006), the role of frataxin protein in mitochondrial function and cell metabolism (Bencze et al., 2006; Calabrese et al., 2005), and the development of anti-oxidants, iron chelators and other chemical modifiers of mitochondrial function as FRDA therapeutics (Boddaert et al., 2007; Lodi et al., 2006; Shults and Haas, 2005); hence, these topics will not be covered here.

2. Analysis of the *FXN* promoter and identification of potential regulatory factors

For the development of many types of *FXN* gene activators, it will be important to identify the cis-acting DNA sequences and trans-acting protein factors that regulate *FXN* gene expression in normal and FRDA cells. Additionally, the influence of chromatin structure on *FXN* gene expression will need to be assessed. This latter topic will be discussed in section 7 below. Three papers have addressed the nature of the DNA sequences and transcription factors responsible

for *FXN* gene regulation. In the first, Usdin and colleagues (Greene et al., 2005) examined human and other primate *FXN* genes for conserved sequences and found repetitive DNA elements (such as SINE and *Alu* sequences) as well as conserved initiator (Inr) and downstream promoter elements (DPE). No TATA sequence is present in the *FXN* upstream region, and thus the Inr and DPE may contribute to basal promoter activity. These authors also performed a promoter deletion analysis and identified retroelement insertions (*Alu* and MIR, mammalian interspersed repeats) as important regulators of *FXN* gene expression, at least in transient transfection reporter gene assays in muscle cell lines. Deletion past the transcription initiation site had the most severe effect on reporter gene activity, reducing activity by 60%, suggesting that downstream elements contribute significantly to *FXN* promoter activity. Gel mobility shift experiments were used to identify protein-binding DNA elements; however, the nature of the protein species responsible for the observed gel-shifts were not identified (Greene et al., 2005). In a subsequent study (Greene et al., 2007), Usdin and colleagues identified an E-box element in the first intron of the *FXN* gene that contributes promoter activity in a luciferase reporter assay. E-box elements are the binding sites for members of the basic-helix-loop-helix family of transcription factors, and gel mobility shift experiments confirmed protein binding to this DNA sequence in nuclear extracts from cultured muscle cells (Greene et al., 2007). One potential binding protein for this element is the muscle-specific factor Mt, and oligonucleotides containing the Mt binding site were shown to compete for the protein-DNA complex. As discussed in more detail below, this region contains sites of DNA methylation that differ between normal and FRDA *FXN* alleles, perhaps suggesting differences in protein occupancy dependent on *FXN* transcriptional activity; however, direct methylation of this site *in vitro* had no effect on reporter gene activity.

In another recent study, Garcia and colleagues examined the consequences of deletion of the *Epas1* gene in mice, encoding the transcription factor Hypoxia-inducible Factor 2alpha (HIF-2 α) (Oktay et al., 2007). These mice exhibit an apparent mitochondrial disease state, similar to knock-outs of the mitochondrial antioxidant enzyme manganese superoxide dismutase (Sod2), and Sod2 was identified as a HIF-2 α target gene. These authors went on to examine the levels of Sod2 in the livers of various knock-out mice, and concluded that factors in addition to Sod2 contribute to the disease like state in *Epas1*/HIF-2 α knock-out mice. Based on a series of biochemical and cell physiology studies, the mouse *FXN* gene was identified as a potential target of HIF-2 α . Based on transient transfection reporter gene assays, the mouse *FXN* promoter was preferentially activated by HIF-2 α , compared to HIF-1 α , through a consensus HIF-responsive enhancer element, located in the upstream region (~1900 bp) of the mouse *FXN* gene. Deletion of the HIF-response element abrogated HIF-2 α activation. Because the upstream regions of the mouse and human *FXN* genes are not well conserved (Greene et al., 2005), it remains to be determined whether HIF-2 α contributes to human *FXN* promoter activity. Additional studies will also be needed to fully describe the protein factors and cis-acting DNA sequences that contribute to *FXN* gene regulation in normal and FRDA cells.

3. Development of cell lines and mouse models for FRDA

To facilitate the identification of molecules to alleviate frataxin deficiency, cell lines and mouse models for FRDA are essential tools for discovery. Although *FXN* knockout mice and cell lines are important tools for understanding the role of frataxin in cell physiology and disease etiology and progression (Bencze et al., 2006; Puccio, 2007; Puccio et al., 2001), these resources are not appropriate for discovery of *FXN* gene activators. For that purpose, several groups have developed transgenic mice harboring *FXN* alleles with expanded GAA·TTC repeats and reporter cell lines. We will first discuss the development of transgenic animal models for FRDA.

Three animal models are currently available for testing the *in vivo* efficacy of potential *FXN* activator molecules: these are the heterozygous [GAA·TTC]₂₃₀ KIKO (knock-in/knockout) and homozygous KIKI (knock-in) mice developed by Pandolfo and colleagues (Miranda et al., 2002), the human YAC *FXN* mouse developed by Pook and colleagues (Al-Mahdawi et al., 2006; Clark et al., 2006), and the *FXN*-EGFP reporter mouse developed by Sarsero and colleagues (Sarsero et al., 2005; Sarsero et al., 2003). In the Pandolfo knock-in mice, a [GAA·TTC]₂₃₀ repeat was inserted into the mouse *FXN* locus at the position of the expansion in human FRDA alleles (*FXN* intron 1). However, this repeat knock-in does not reproduce an overt FRDA-like phenotype, although it does result in decreased frataxin RNA and protein (Miranda et al., 2002). Only after 12 months of age do the KIKO mice develop mild locomotor symptoms (M. Pandolfo, personal communication). Nonetheless, the *FXN* knock-in mouse provides an appropriate animal model for testing the *in vivo* efficacy of transcriptional activators: the KIKO mouse exhibits between 25 – 36% of wild-type *FXN* mRNA and protein in various organs, compared to wild-type mice (Miranda et al., 2002). These values lie within the range observed in mildly affected FRDA patients. In contrast, the KIKI mouse exhibits ~65% of WT *FXN* mRNA in various tissues. Thus, compounds that increase *FXN* transcription can be assayed *in vivo* with these mouse models.

To circumvent the limitations of the knock-in mouse model, Pook and colleagues have generated two lines of human *FXN* YAC transgenic mice, designated YG22 and YG8, containing 190 and 190+90 repeat expansions, respectively (Al-Mahdawi et al., 2006). Both transgenic lines exhibit intergenerational and age-related somatic instability of the repeat, with the most prominent expansions occurring specifically in the cerebellum (as seen in FRDA autopsy tissues) and in the dorsal root ganglia (DRG) (Al-Mahdawi et al., 2006; Clark et al., 2006). By cross breeding the *FXN* YAC transgenic mice with heterozygous *FXN* knockout mice from Puccio and colleagues, the Pook laboratory has further shown that both transgenes are able to successfully rescue homozygous *FXN* knockout embryonic lethality and that the rescue mice exhibit an FRDA-like phenotype (Al-Mahdawi et al., 2006). In particular, these mice exhibit decreased *FXN* mRNA and frataxin expression in affected organs, decreased coordination ability (as determined by rotarod testing), decreased aconitase activity, oxidative stress, histopathology in DRG large sensory neurons and iron accumulation in the heart. Therefore, this is an excellent FRDA mouse model in which to investigate the potential therapeutic effects of transcriptional activators.

Sarsero and colleagues have taken a different approach toward generating a mouse model and cell lines derived from these mice for compound screening (Sarsero et al., 2005; Sarsero et al., 2003). These investigators generated transgenic mice with a BAC genomic reporter construct consisting of an in-frame fusion between the human *FXN* gene and the gene coding for enhanced green fluorescent protein (EGFP). As such, expression of EGFP is under control of the *FXN* promoter, and compounds that upregulate *FXN* promoter activity might be identified with these animals. Note, however, that this construct does not contain expanded GAA·TTC repeats (6 repeats, as found in healthy individuals), and therefore only allows for the identification of molecules that act on the wild-type promoter and not on the repeats. Production of full-length frataxin-EGFP fusion protein from this construct was demonstrated by immunoblotting. EGFP expression was observed as early as day E3.5 of development and most tissues of adult transgenic mice were fluorescent. Stable cell lines from the full-length *FXN*-EGFP fusion were generated and mitochondrial fluorescence was observed in these cells, suggesting proper sub-cellular localization of the fusion protein (Sarsero et al., 2003). The authors also tested two compounds for their effects on EGFP production by flow cytometry and both hemin and butyric acid were found to have small positive effects (less than 20% above basal levels). Currently, these reporter cell lines are being used for screening large numbers of compounds for increased *FXN* transcription (J. Sarsero, personal communication).

Hebert and colleagues have generated an additional GFP reporter cell line that will be useful for compound screening (Grant et al., 2006). These authors fused part of the first intron of *FXN*, containing either 15 or 148 GAA·TTC repeats, to the coding sequence for EGFP (in the Clontech plasmid pEGFP-N3), under control of the human cytomegalovirus immediate early promoter, and established stable HeLa cell lines. Both fluorescence microscopy and western blotting with antibodies to GFP demonstrated reduced levels of GFP expression in the [GAA·TTC]₁₄₈ cell line, compared to the [GAA·TTC]₁₅ cell line (Grant et al., 2006), showing that these are appropriate cell lines in which to screen for compounds that alleviate GAA·TTC-mediated silencing.

Finally, the National Institutes of General Medical Sciences Human Genetic Cell Repository (Coriell Institute, New Jersey) has available a collection of established lymphoblast and fibroblast cell lines derived from FRDA patients, their parents and siblings. *FXN* alleles with different repeat numbers are represented in this collection (see <http://www.coriell.org/nigms>; search FRDA). These cells are useful for assessing the efficacy of compounds that act on the endogenous *FXN* gene in its native chromosomal context, which may be of importance if chromatin structure is a major determinant of *FXN* gene activity (discussed below).

4. Discovery of molecules that increase frataxin mRNA or protein

There are several reports of molecules that increase frataxin mRNA or protein levels. These include cisplatin (Ghazizadeh, 2003), 3-nitropropionic acid (Turano et al., 2003), sodium butyrate and hemin ((Sarsero et al., 2003), see above), and erythropoietin (Sturm et al., 2005). In the case of the chemotherapeutic agent cisplatin, Ghazizadeh discovered through a cDNA microarray analysis that the frataxin gene was often over-expressed in cisplatin-resistant cell lines, compared to parental cell lines. This finding may be related to the role of frataxin in mitochondrial homeostasis and detoxification of reactive oxygen species (ROS). Given the harsh side effects of this potent chemotherapeutic, it is unlikely that cisplatin will be considered an attractive therapeutic for FRDA. In a separate study, Turano et al., investigated the effects of agents that induce mitochondrial oxidative stress on frataxin expression. These authors reported that 3-nitropropionic acid (3-NP) increases frataxin mRNA and protein in both FRDA lymphoblasts and lymphoblasts from normal individuals in culture, but millimolar concentrations were required to elicit this effect. 3-NP irreversibly inhibits succinate dehydrogenase, causing the generation of ROS by interrupting the complex II electron transfer cascade. Increases in *FXN* transcription by 3-NP may be related to the observation that cisplatin induces *FXN* expression, and that *FXN* expression is increased as a stress response. Finally, recombinant human erythropoietin (rhuEPO) has been reported to increase levels of frataxin protein in lymphocytes from FRDA patients, and in various cell lines in culture (Sturm et al., 2005). The molecular basis for this observation has not been established, but might be related to increased translation or stabilization of frataxin protein. In earlier studies, rhu-EPO was shown to cross the blood-brain barrier in a human pharmacokinetics study (Xenocostas et al., 2005) and in an animal model for brain injury (Brines et al., 2000). Clinical trials with rhuEPO are currently in progress.

5. Other therapeutic strategies for FRDA

Based on the two models for gene silencing by expanded GAA·TTC repeats, molecules might be identified that either stabilize normal B-type DNA or revert heterochromatin and thus reactivate silenced FRDA alleles. Rational selection and screening of potential therapeutic molecules is one approach to identify such molecules (Grant et al., 2006; Herman et al., 2006; Sarsero et al., 2005), or alternatively, compound libraries might be screened in a high throughput fashion with the fluorescent reporter cell lines described above. To date, high throughput screens have not been reported; however, this approach might lead to the

identification of molecules that increase *FXN* promoter activity, or elongation through the *FXN* gene, or act at other steps in the transcription process. High throughput screens are unbiased as to the molecular mechanism(s) responsible for silencing and could yield important information on the silencing mechanism. Rational selection of molecules that might affect DNA structure has been reported (Burnett et al., 2006; Grabczyk and Usdin, 2000a; Grant et al., 2006) and the results of these studies will be reviewed below. Additionally, molecules that alter chromatin structure by changing the postsynthetic modification states of the histone proteins, namely histone deacetylase inhibitors, have been screened for their effects on *FXN* gene expression (Herman et al., 2006; Sarsero et al., 2003).

6. DNA ligands to increase *FXN* gene expression

Wells and colleagues (Napierala et al., 2004) and Grabczyk and Usdin (Grabczyk and Usdin, 2000a) proposed that DNA ligands that would cause a transition from triplexes and/or sticky DNA to normal B-type DNA might restore transcription to *FXN* alleles harboring long repeats. From the results of *in vitro* transcription experiments with T7 RNA polymerase, Grabczyk and Usdin proposed a model where an intramolecular RRY DNA triplex is formed behind an advancing RNA polymerase on DNA templates containing long GAA·TTC repeats (Grabczyk and Usdin, 2000b). These authors predicted that agents that inhibit formation of this triplex during transcription would increase successful elongation through GAA·TTC tracts. In a subsequent report, oligodeoxyribonucleotides were designed to block RRY triplex formation by GAA·TTC repeats, and were found to provide specific and concentration-dependent increases in full-length transcripts. (Grabczyk and Usdin, 2000a). Thus, DNA-binding agents have the potential to overcome transcription silencing at pathogenic *FXN* alleles. While these oligonucleotides relieve GAA·TTC-mediated gene silencing in this simple *in vitro* system, nucleic acids suffer from poor cell permeability. Thus delivery strategies, such as viral vectors or liposomes, must be used for effective nucleic acid gene targeting *in vivo*. Therefore, cell permeable small molecules that interfere with triplex formation at GAA·TTC repeats might prove more effective than oligonucleotides in treating FRDA. Small molecules that prefer duplex DNA over triplexes or other structures would disfavor the transition from B-type DNA to triplexes of the type found in pathogenic FRDA alleles (see (Sakamoto et al., 1999) and references therein), which necessitate duplex melting concomitant with triplex formation. One important consideration, however, is that the molecules themselves should not prevent transcription elongation by RNA polymerase II.

Two groups have now focused on the identification of small DNA ligands to alleviate GAA·TTC repeat-mediated gene silencing. Hebert and colleagues (Grant et al., 2006) recently reported the results of a rational screen of small molecule DNA ligands for their effects on EGFP expression in the reporter cell lines described above. This study was based on the results of a screen of compounds for their ability to discriminate between different nucleic acid structures in a competition dialysis experiment (reviewed in (Chaires, 2005)). In this study, 126 compounds were screened for their relative affinities for 13 different nucleic acid structures, and compounds that were found to prefer duplex DNA compared to triplex or other structures were then chosen for assay in the expanded *FXN*-EGFP cell line (Grant et al., 2006). While some of the tested compounds were cytotoxic, some compounds such as DAPI, diminazene, Hoeschst 33258, distamycin and pentamidine were found to increase GFP expression in the [GAA·TTC]₁₄₈-EGFP cell line, quite possibly by displacing the third strand of a triplex and restoring the DNA back to duplex. Importantly, the anti-infective pentamidine (Figure 1), which is used in the treatment of HIV-associated opportunistic infections, was shown to increase frataxin protein in primary lymphocytes isolated from FRDA donor blood by 1.3 to 2.2-fold over untreated lymphocytes. In recent work, Hebert and colleagues examined the activities of a small library of Hoeschst 33258 derivatives for DNA binding properties and transcription in the [GAA·TTC]₁₄₈-EGFP reporter cell line (Whittom et al., 2007). This group

identified additional aromatic amidines that increase GFP expression in cells, without affecting the activity of the [GAA·TTC]₁₅-EGFP control reporter. These molecules are minor groove binders that prefer A-T rich duplex DNA over other DNA structures. These studies are an important demonstration of the utility of small molecules for activating *FXN* gene transcription both in reporter systems and at the endogenous locus in patient cells, but additional studies will be needed to assess whether these compounds function in animal models for FRDA. It will also be important to assess the mechanism(s) by which these compounds activate *FXN* transcription.

In addition to the molecules screened by Hebert and colleagues, a number of other natural and synthetic ligands have been identified that possess the ability to bind duplex DNA (Gottesfeld et al., 2000; Wemmer and Dervan, 1997). Among these molecules, the pyrrole-imidazole polyamides are unique in that they can be programmed to bind predetermined DNA sequences (Dervan and Edelson, 2003). Rules to rationally design minor-groove-binding polyamides containing *N*-methylimidazole (Im), *N*-methylpyrrole (Py), 3-hydroxy-*N*-methylpyrrole (Hp) and β-alanine (β) amino acids have been established (Dervan, 2001; Dervan and Edelson, 2003). Two classes of polyamides are available for gene targeting: hairpin polyamides that bind mixed sequence DNA with high affinity and specificity (Dervan, 2001; Trauger et al., 1996) and linear β-alanine linked polyamides that bind to homopurine stretches of DNA (Janssen et al., 2000b; Urbach and Dervan, 2001). Thus, for DNA sequences such as GAA·TTC repeats, linear β-linked polyamides offer promise for effective targeting (Janssen et al., 2000b; Urbach and Dervan, 2001), and such molecules have been shown to bind several different homopurine sequences *in vitro* (Urbach and Dervan, 2001) and GAGAA repeats in *Drosophila* satellite DNA both *in vitro* and in *Drosophila* embryos (Janssen et al., 2000b). These satellite DNA-specific molecules induce chromatin opening and reverse heterochromatin-mediated position effect gene silencing when administered to *Drosophila* embryos (Janssen et al., 2000a; Janssen et al., 2000b), suggesting that similar molecules that target GAA·TTC DNA could reverse *FXN* gene silencing. Figure 2 shows the structure of a β-linked polyamide bound to a 9 bp homopurine run of DNA, similar to GAA·TTC repeat DNA (Urbach et al., 2002). Given the very high affinity of polyamides for their target sites, these molecules should act as a thermodynamic “sink” and lock GAA·TTC repeats into double stranded B-type DNA, preventing the transition to non-B DNA structures proposed for long GAA·TTC repeats.

Important considerations for development of small molecule DNA therapeutics for FRDA are cross-reactivity with other DNA sequences, cell permeation, nuclear localization, gene targeting in nuclear chromatin, and bioavailability and toxicity *in vivo*. Each of these parameters has been investigated for Py-Im polyamides, at least in cell culture and in mouse models for human disease (reviewed in (Dervan and Edelson, 2003)). Polyamides bound within coding regions of genes are without effect on transcription in mammalian cells, suggesting that polyamides bound within GAA·TTC repeat DNA should not affect transcriptional elongation. Finally, polyamides do not bind either single stranded or duplex regions of RNA. Hence, these molecules should have no effect on RNA processing or translation of frataxin mRNA.

Based on the NMR structure presented by Urbach *et al.*, ((Urbach et al., 2002), and Figure 1), polyamide FA1, ImPyβImPyβImβDp (Figure 3), was synthesized in this author's laboratory to target the nine base pair sequence 5'-AAGAAGAAG-3', as in GAA·TTC repeat DNA (Burnett et al., 2006). DNase I footprinting under equilibrium conditions was used to monitor the binding affinity of this molecule with a DNA fragment harboring six GAA·TTC repeats, and a $K_a = 1 \times 10^{10} \text{ M}^{-1}$ ($K_d = 100 \text{ pM}$) was obtained. Changing the sequence of Im and Py amino acids, without changing the chemical composition of the molecule (resulting in FA2, ImPyβImImβPyβDp), reduces binding affinity by greater than three orders of magnitude (K_d

= >100 nM). Longer polyamides to target extended regions of GAA·TTC repeat DNA were also synthesized (FA3 and FA4, Figure 3).

As discussed above, for polyamides to be useful as human therapeutics, they must be cell permeable and transit to the nucleus. Fluorescent versions of each of the match polyamides were synthesized, where the dye Bodipy FL was attached at the carboxyl terminus of the polyamide (Figure 3), and each of the polyamide-bodipy conjugates localizes in the nucleus of live, unfixed FRDA lymphoblast cells in culture (Burnett et al., 2006). To assess whether the GAA-specific polyamides reverse the transcription defect caused by expanded GAA·TTC repeats in the frataxin gene, we used quantitative real time/reverse transcriptase PCR (qRT-PCR) to monitor frataxin mRNA levels in FRDA lymphoblasts. A cell line from a Friedreich patient (from the NIGMS Coriell collection) had a markedly lower level (6 – 13%) of frataxin mRNA compared to a cell line from an unaffected sibling, and on addition of polyamides, we found that polyamide FA1 increased *FXN* transcription ~2 to 3-fold (at 2 μ M), but no effects were found with any of the other polyamides. Similar incubations with the mismatch polyamide FA2 only had a modest effects on *FXN* transcription. A ~2 – 3-fold increase in frataxin protein was observed with FA1 in the FRDA cells, which correlates well with the observed increase in *FXN* mRNA. Our data are thus consistent with the expectation that polyamide FA1 has the desired effect of reversing inhibition of *FXN* transcription. We also examined the effects of the GAA-specific polyamides on global gene expression in the lymphoid cell lines using Affymetric high-density DNA microarrays. Surprisingly, only a limited number of genes are significantly affected in FRDA cells (at $P = 0.005$, 51 genes affected by 1 μ M FA1, and 16 genes affected by 2 μ M FA1; (Burnett et al., 2006)), and many of these genes contain binding sites for the polyamides, suggesting that these changes in gene expression are a direct consequence of polyamide binding to their target sites.

Polyamides also reversed the sticky DNA conformation adopted by GAA·TTC repeats in supercoiled plasmid DNA (Burnett et al., 2006). Up-regulation of *FXN* transcription with GAA·TTC-specific polyamides may be due to a change in DNA structure, as suggested by reversal of “sticky” DNA, which then allows for transcription through expanded GAA·TTC-repeat DNA, or by reversing heterochromatin caused by expanded GAA·TTC repeats (Saveliev et al., 2003). Polyamides designed to target the related DNA sequence 5'-AAGAG-3', which is found in *Drosophila* satellite V DNA, have been shown to alter gene expression in developing embryos by opening the chromatin domains containing these repeated sequences (Janssen et al., 2000b; Saveliev et al., 2003) and to displace heterochromatin protein 1 (HP1) and the other chromosomal proteins D1 and topoisomerase II from these sequences (Blattes et al., 2006). Interestingly, HP1 was found to promote gene silencing by expanded GAA·TTC-repeat DNA (Saveliev et al., 2003). Thus, polyamides targeting GAA·TTC-repeat DNA may increase transcription of genes that harbor these sequences by displacement of similar repressor proteins, and reversal of inactive heterochromatin to a more active chromatin structure. Additional studies will be needed to elucidate the mechanism whereby polyamides up-regulate *FXN* transcription in living cells.

7. Chromatin structure of the frataxin gene

To assess the heterochromatin model for gene silencing by expanded GAA·TTC repeats, our laboratory examined histone modifications on *FXN* gene chromatin using chromatin immunoprecipitation (ChIP) methods, with antibodies to various histone modifications and cell lines derived from FRDA patients. Primers that interrogate the chromatin regions upstream or downstream of the GAA·TTC repeats in the first intron of the *FXN* gene, as well as the promoter element, were used in the ChIP experiments, with the levels of immunoprecipitated DNA quantified by real-time PCR (Herman et al., 2006). No significant differences in the levels of histone acetylation were found on the *FXN* promoter in cell lines from an FRDA

patient and his/her unaffected sibling. However, the coding region of active *FXN* alleles in the normal cell line is enriched in histones acetylated at H3-K9, H3-K14, H4-K5, H4-K8, H4-K12, and H4-K16, compared to the inactive alleles in the FRDA cell line, which are significantly depleted in these histone modifications. Additionally, H3-K9 is highly trimethylated in the FRDA cell line compared to the normal cell line. Along with hypoacetylation, trimethylation of H3-K9 is a hallmark of heterochromatin (Elgin and Grewal, 2003). Recent unpublished studies in the KIKI mouse model also suggest hypoacetylation and H3-K9 trimethylation at repressed *FXN* alleles (M. Rai and M. Pandolfo, personal communication). Thus, the histone postsynthetic modification state within the coding region of inactive *FXN* alleles is consistent with a chromatin-mediated mechanism as the cause of gene silencing in FRDA (Saveliev et al., 2003).

A recent study examined the extent of DNA methylation on active versus inactive *FXN* alleles in cell lines (Greene et al., 2007). Along with histone hypoacetylation and particular sites of histone methylation (e.g., H3K9), DNA methylation is also a hallmark of gene repression. Indeed, DNA methylation is a mark for recruitment of repressor complexes containing histone deacetylases, mediated by methyl-CpG binding proteins (El-Osta and Wolffe, 2000). Usdin and colleagues used bisulfite mapping to examine the distribution of methylated cytosine residues in the DNA region adjacent to the GAA·TTC repeats, and found that the *FXN* gene was methylated in both unaffected and affected individuals, with more extensive methylation in patient cells (Greene et al., 2007). Specifically, three cytosine residues were almost completely methylation-free in the *FXN* genes in unaffected individuals but almost always methylated in FRDA patient DNA. One of these residues is located within an E-box, which is the binding site for members of the basic-helix-loop-helix family of transcription factors. Deletion of this E-box sequence caused a significant drop in promoter activity in reporter assays, demonstrating the importance of this sequence for *FXN* promoter activity. These data also suggest that repeat-mediated changes in DNA methylation may also affect transcription initiation by blocking binding of factors that increase *FXN* promoter activity. Elevated levels of histone H3 dimethylated on lysine 9 were also seen in FRDA cells, consistent with a more repressive chromatin organization (Herman et al., 2006; Saveliev et al., 2003). Similar DNA methylation results have been found by Pook, both in his *FXN*-YAC mouse model and in human FRDA tissue samples (M. Pook, personal communication).

Based on these results and those of Festenstein and colleagues (Saveliev et al., 2003), a model for chromatin-mediated silencing of the *FXN* gene by expanded GAA·TTC repeats involves the initial recognition of the repeats by either direct protein recognition of the DNA sequence or the non-B DNA structure adopted by expanded GAA·TTC repeats, followed by a cascade of events that lead to chromatin condensation. The nature of the protein species responsible for DNA recognition remains to be determined. Alternatively, an RNA-mediated silencing mechanism is also possible (Bernstein and Allis, 2005), although no published studies have addressed this mechanism in FRDA gene silencing. Once targeted for silencing, HDACs are recruited to *FXN* gene chromatin, perhaps through DNA methylation; these enzymes deacetylate the amino-terminal tails of the histones on *FXN* gene chromatin; a histone methyl transferase (HMT) then trimethylates H3K9, and because HP1 proteins bind H3K9me3, they are then recruited to expanded *FXN* gene chromatin. All of these events lead to chromatin condensation and *FXN* gene silencing. Based on this model, we speculated that HDAC inhibitors will reverse *FXN* silencing by directly increasing acetylation at particular residues, which will lead to displacement of HP1 and chromatin decondensation.

8. Histone deacetylase inhibitors as potential therapeutics for FRDA

Acetylation and deacetylation of histone proteins, as well as other proteins involved in transcriptional regulation (such as DNA-binding transcription factors, nuclear hormone

receptors, and signal transduction proteins), play critical roles in regulating gene expression. Chromatin structural changes and histone deacetylation are involved in various neurological diseases (Kernochan et al., 2005; Langley et al., 2005), including the triplet-repeat diseases myotonic dystrophy and Fragile X mental retardation (Cho et al., 2005; Tapscott et al., 1998), and in spinal muscular atrophy (Kernochan et al., 2005). Thus, HDAC inhibitors may revert silent heterochromatin to an active chromatin conformation, and restore the normal function of genes that are silenced in these diseases (Di Prospero and Fischbeck, 2005). A number of studies have focused on the use of HDAC inhibitors as therapy for restoring histone acetylation and transcriptional activation in cell culture studies and *in vivo* neurodegenerative mouse models (Langley et al., 2005). HDAC inhibitors have been shown to cross the blood-brain barrier, to ameliorate motor deficits in mouse models for Huntington's disease (Ferrante et al., 2003; Gardian et al., 2005; Hockly et al., 2003), and to increase expression of the SMN2 gene in spinal muscular atrophy (Kernochan et al., 2005; Riessland et al., 2006). Thus, interest in this class of compounds as therapeutics for other neurological diseases has emerged (Di Prospero and Fischbeck, 2005).

Based on the hypothesis that the chromatin structure and histone modification state of the *FXN* gene is responsible for gene repression on alleles containing expanded GAA·TTC repeats, we asked whether histone deacetylase inhibitors might affect transcription of the silenced *FXN* gene. An earlier study demonstrated a small effect of the general HDAC inhibitor sodium butyrate on *FXN* gene activity in the EGFP reporter cell line (Sarsero et al., 2003). Our results (Herman et al., 2006) indicate that a commercially available histone deacetylase inhibitor (BML-210, from BioMol; Figure 4), and derivatives we have synthesized, relieve repression of the *FXN* gene in both lymphoid cell lines derived from FRDA patients and in primary lymphocytes from donor FRDA patient blood. We also observe increases in frataxin protein in cells treated with our HDAC inhibitors that quantitatively parallel the observed changes in *FXN* mRNA. This finding is important since it demonstrates that expanded GAA repeats in the primary *FXN* RNA transcript do not interfere with RNA processing. Surprisingly, we find that only members of the benzamide family of HDAC inhibitors related to BML-210 reactivate the silenced *FXN* gene in FRDA cells. For example, the related hydroxamic acid SAHA (Figure 4) is inactive as an activator of *FXN* transcription in FRDA cells, although SAHA is a superior general HDAC inhibitor than BML-210 and derivatives. Inspection of microarray data for SAHA (Peart et al., 2005), trichostatin A and sodium butyrate (available at the NIH GEO website, <http://www.ncbi.nlm.nih.gov/projects/geo>, search FRDA and histone deacetylase inhibitor) indicate that these common HDAC inhibitors have no positive effect on *FXN* transcription in non-FRDA cells. Taken together, these results suggest that a specific HDAC, or HDAC-protein complex, is involved in *FXN* gene silencing and only members of the BML-210 family are active against this HDAC in cells; efforts are underway to identify this enzyme and its mechanism of action in *FXN* gene silencing.

To improve on the activity of BML-210, we explored the length of the linker region between the two ring systems (four, five and six methylenes), the nature of the rings (phenyl, pyridine, quinoline), and the type and position of ring substituents (amino, methyl and methoxy groups, etc., Table 1 of (Herman et al., 2006)). Compounds with six or four methylene groups in the linker region are less potent than the corresponding pimeloylanilide derivatives (for example, compound **4b**, *N*¹-(2-aminophenyl)-*N*⁷-phenylheptanediamide (Wong et al., 2003), Figure 4), and amino- or methoxy-substitutions at the ortho- and para- positions are most effective in increasing levels of *FXN* mRNA. The quinoline derivatives of pimeloylanilide are also highly active. We will refer to this class of compounds as pimelic benzamides. We find no significant correlation between HDAC inhibition activity and the ability of the compounds to activate *FXN* transcription in FRDA cells, in accord with the finding that highly active class I/II HDAC inhibitors, such as TSA or SAHA, have no effect on *FXN* mRNA levels. Since nuclear extracts contain numerous HDAC enzymes, the HDAC inhibition assay provides only an overall

measure of inhibitory activity for the sum of all these enzymatic activities, and this assay likely does not reflect the true IC_{50} for the target enzyme(s) involved in *FXN* gene silencing in FRDA.

Since frataxin deficiency in the human disease affects non-proliferating cell types (neuronal cells and cardiomyocytes), we assayed our compounds in primary human lymphocytes isolated from normal or FRDA patient donor blood. Primary lymphocytes can be maintained in culture for several days and un-stimulated lymphocytes do not divide, providing a surrogate model for terminally differentiated cells. We find that our compound **4b** increased the levels of *FXN* mRNA in each of the tested lymphocyte populations in numerous experiments, and the level of *FXN* mRNA in the FRDA patient lymphocytes was increased to approximately that of the carrier. Notably, *FXN* mRNA was nearly doubled in lymphocytes from heterozygous carrier individuals, suggesting that the inactive *FXN* allele can be nearly completely re-activated. Since heterozygous individuals do not exhibit FRDA symptoms, we believe our compounds have elicited a therapeutically useful increase in *FXN* mRNA in patient cells.

We also asked whether the HDAC inhibitors directly modify the histone acetylation state of *FXN* gene in cellular chromatin. To answer this question, we again performed ChIP experiments with antibodies to the acetylated forms of histones H3 and H4 in both the FRDA and normal cell lines. We treated FRDA cells with HDAC inhibitor **4b** and analyzed histone acetylation on the chromatin region immediately upstream of the repeats. Similar ~2.5 to 3-fold increases in *FXN* transcription and acetylation at H3-K14, H4-K5 and H4-K12 are observed with **4b** in these cells. No significant changes in acetylation are observed at other amino acid residues in histones H3 or H4 on *FXN* gene chromatin. As a control for this experiment, we repeated the ChIP assays with cells that had been treated with the common HDAC inhibitors SAHA and TSA. Neither compound affected *FXN* mRNA levels or histone acetylation on the *FXN* gene (Herman et al., 2006). These data suggest that **4b** directly inhibits an HDAC enzyme associated with the *FXN* gene, thus resulting in increased levels of acetylated histones by the action of an associated histone acetyltransferase, ultimately leading to increases in *FXN* transcription. A model for *FXN* gene silencing and gene reactivation by **4b** is shown in Figure 5. The finding that acetylation levels at only particular lysine residues are increased with the HDAC inhibitor **4b** may be related to a particular histone modification code (Strahl and Allis, 2000) that regulates activity of the *FXN* gene. Biochemical and electron microscopy studies of nucleosomal arrays indicate that acetylation of histone amino-terminal tails prevents chromatin condensation (Annunziato et al., 1988; Annunziato and Hansen, 2000; Shogren-Knaak et al., 2006; Tse et al., 1998) and may be directly related to gene activation (Tse et al., 1998); hence histone acetylation and concomitant chromatin fiber decondensation are likely prerequisites for *FXN* transcription. We speculate that our HDAC inhibitors directly affect an HDAC(s) that deacetylates H3K14, H4K5 and H4K12, and these residues are then acetylated by the action of an associated HAT, leading to displacement of HP1 and concomitant opening of the chromatin fiber for *FXN* transcription. Notably, published studies have documented that acetylation of H3K14 can lead to displacement of HP1 (Mateescu et al., 2004), in agreement with our model for *FXN* gene silencing (see above).

We find that the pimelic benzamide HDAC inhibitors increase total histone acetylation in the mouse brain when administered by either subcutaneous or intravenous injection (as assayed by western blotting of total brain histones; D. Herman and J.G., unpublished), suggesting that these molecules cross the blood brain barrier and function as HDAC inhibitors *in vivo*. Moreover, the compounds exhibit no obvious acute toxicity in these Balb/c mice. Recent studies in the laboratory of M. Pandolfo demonstrate that compound **4b** and derivatives increase *FXN* transcription in the brain and cerebellum of KIKO and KIKI mouse models (M. Rai and M. Pandolfo, personal communication). Full pharmacokinetic and toxicity studies of these molecules are underway.

9. Other chromatin approaches toward gene activation

A recent paper suggests a novel approach towards gene activation, by inhibiting the lysine-specific histone demethylase LSD1 (Huang et al., 2007). Previous studies have established that histone H3K4me2 is an active chromatin signature at gene promoters, and demethylation of H3K4 by LSD1 may repress gene expression (reviewed in (Huang et al., 2007)). Since LSD1 is a homologue of the FAD-dependent polyamine oxidases, polyamine analogues, such as guanidines that are known to inhibit this enzyme family, may also be inhibitors of LSD1. Consistent with this expectation, Huang et al. report that biguanide and bisguanidine polyamine analogues are potent inhibitors of LSD1, and cause the re-expression of genes that are silenced in human colon carcinoma cells, including members of the secreted frizzles-related proteins (SFRPs) and the GATA family of transcription factors. These authors used ChIP analysis to demonstrate that gene activation coincides with increased H3K4me2 and acetyl-H3K9, and decreased H3K9me1 and H3K9me2 repressive marks at the SFRP and GATA genes. SFRP and GATA gene activation and increased H3K4me2 were also observed with siRNAs to LSD1 (although the polyamine analogues were quantitatively more effective than siRNAs), providing evidence for the mechanism of action of the inhibitors. These new agents may thus be useful for reversing aberrant repression of gene transcription, and could well lead to another line of therapeutics for FRDA and related gene-silencing diseases.

10. Concluding remarks and open questions

With the development of reporter cell lines and transgenic mouse models, the FRDA research community is awaiting the results of high throughput screens for molecules that will increase *FXN* gene expression, and the disclosure of the structures of such molecules. As mentioned above, high throughput screens of large compound libraries are unbiased as to mechanism (except for the bias introduced by the design of the assay screen itself), and the identity of the “hits” from such screens may give us additional important information on the mechanisms of gene silencing and regulation of the *FXN* gene in Friedreich ataxia. Such information will be important for further therapeutic development. The exciting findings from Hebert and colleagues (Grant et al., 2006) that common small DNA binding molecules increase *FXN* gene activity provides a solid starting point for further synthesis and screening, in the hope that more specific and less toxic molecules can be identified. Additionally, identification of the HDAC inhibitor target, that is the actual enzyme or enzymes that are inhibited by the pimelic benzamide HDAC inhibitors, will provide still more information on the silencing mechanism.

Finally, just how expanded repeats induce silencing remains an open question. There is no doubt that expanded GAA·TTC repeats can form alternative DNA structures both *in vitro* and in plasmid DNAs transfected into cells, and so the important question remains, do these structures actually exist on FRDA *FXN* alleles in cells? If so, is it this structure that dictates silencing, by recruiting heterochromatin proteins, HDACs, etc? Or, is it simply the repetitive nature of long GAA·TTC repeats, as duplex DNA, that initiates silencing? Are there specific DNA binding proteins that recognize GAA·TTC repeats (Maueler et al., 1999) and initiate silencing? Could the microRNA or RNAi pathway be involved in silencing, through heterochromatin formation? A link between histone H3K9 methylation and RNA-mediated gene silencing has been proposed (reviewed by (Bernstein and Allis, 2005)). While there is ample data in the literature to establish that repetitive DNA induces gene silencing (see (Saveliev et al., 2003)), the biochemical mechanisms are not fully elucidated for repetitive DNA-mediated silencing. Thus, we are only at the beginning of discovery of therapeutic strategies for FRDA based on increased *FXN* transcription, and understanding the molecular basis for gene silencing in this disease.

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Abbreviations

FXN, the frataxin gene; FRDA, Friedreich ataxia; HDAC, histone deacetylase.

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Figure 1. Structure of the DNA-binding molecule pentamidine. Taken from the PubChem data base (<http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=4735>).

Figure 2.

Stereo view of an ensemble of 12 NMR structures of the complex of ImPy β Im β ImPy β Dp (shown in gold) bound to 5'-AAAGAGAAG-3' (DNA in blue). Im = imidazole, Py = pyrrole, β = β -alanine, and Dp = dimethylaminopropylamide. The polyamide binds in the 3' to 5' orientation with respect to the N $\rightarrow$ C direction of the ligand. The DNA geometry is fully B-type, with a narrow minor groove. Figure taken from (Urbach et al., 2002), with permission.

Figure 3.

Polyamide structures and binding models. **(A)** Structures of polyamides FA1, FA2, FA3, FA5 and FA6, and the bodipy conjugate of FA1, FA1-Bodipy. Polyamides FA3, FA5, FA6 (ImPyβ(ImPyβ)₂₋₄ImβDp) are designed to target longer GAA·TTC repeats ([GAA]₄₋₆) as shown for FA3 in panel B. **(B)** Polyamide structures are represented schematically as binding models, with open circles representing Py; filled circles, Im; diamonds, β-alanine. Mismatches formed with polyamides FA2 and FA4 are indicated with shaded boxes. Figure adapted from (Burnett et al., 2006).

Figure 4.
Structures of SAHA, BML-210 and **4b** (Herman et al., 2006).

Figure 5.

A model for reactivation of *FXN* transcription by histone deacetylase inhibitor **4b**. HDACs deacetylate the amino terminal tails of the histones, promoting histone methylation at H3K9 and recruitment of heterochromatin proteins, resulting in *FXN* chromatin condensation and gene silencing. HDAC inhibitor **4b** reverses silencing by inhibiting HDACs leading to histone acetylation by the action of associated histone acetyltransferases (HATs) and opening the chromatin domain containing the *FXN* gene. Taken from (Festenstein, 2006), with permission.