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Lipophilic methylene blue analogues enhance mitochondrial function and increase frataxin levels in a cellular model of Friedreich's ataxia

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ABSTRACT

Friedreich's ataxia (FRDA) is an autosomal recessive neurodegenerative disorder resulting from reduced expression of the protein frataxin (FXN). Although its function is not fully understood, frataxin appears to help assemble iron sulfur clusters; these are critical for the function of many proteins, including those needed for mitochondrial energy production. Finding ways to increase FXN levels has been a major therapeutic strategy for this disease. Previously, we described a novel series of methylene violet analogues and their structural optimization as potential therapeutic agents for neurodegenerative and mitochondrial disorders. Presently, a series of methylene blue analogues has been synthesized and characterized for their *in vitro* biochemical and biological properties in cultured Friedreich's ataxia lymphocytes. Favorable methylene blue analogues were shown to increase frataxin levels and mitochondrial biogenesis, and to improve aconitase activity. The analogues were found to be good ROS scavengers, and able to protect cultured FRDA lymphocytes from oxidative stress resulting from inhibition of complex I and from glutathione depletion. The analogues also preserved mitochondrial membrane potential and augmented ATP production. Our results suggest that analogue **5**, emerging from the initial structure of the parent compound methylene blue (MB), represents a promising lead structure and lacks the cytotoxicity associated with the parent compound MB.

1. Introduction

Mitochondrial dysfunction and oxidative damage are hallmarks of numerous neurodegenerative diseases such as Friedreich's ataxia (FRDA), as well as Alzheimer's and Parkinson's diseases.[1–5] FRDA is an autosomal recessive neurodegenerative disease usually caused by large homozygous expansions of a GAA trinucleotide repeat in the first intron of the *FXN* gene, impairing expression levels of fully functional frataxin protein by formation of sticky DNA and triple helices.[6–10] FXN protein levels in individuals with FRDA are inversely correlated with the GAA repeat-burden in the *FXN* gene.[7,9] A large number of GAA repeats is associated with lower FXN protein levels, an earlier age-of-onset of FRDA and a faster rate of disease progression.[9] Frataxin is encoded in the nucleus, expressed as a precursor polypeptide in the cytoplasm and imported into mitochondria with an important role in respiratory function and iron homeostasis.[11–16] FRDA is strongly associated with reduced synthesis of this mitochondrial iron chaperone, leading to mitochondrial iron accumulation, dysfunction of mitochondrial Fe-S containing enzymes (in particular mitochondrial respiratory complexes I, II and III as well as mitochondrial and cytosolic aconitases[17]) and increased Fenton-mediated free radical production.[1,4]

Methylene blue (MB) is a redox active agent with a long history as a therapeutic agent. Clinical applications have included treatment of malaria, cyanide poisoning, methemoglobinemia and ifosfamide-induced encephalopathy.[18–21] MB is widely recognized as being neuroprotective in many neurodegenerative diseases such as Alzheimer's disease.[22] As a redox active agent, MB is able to cycle readily between its oxidized and reduced forms, enabling redirection of electrons to the mitochondrial electron transport chain, thereby enhancing adenosine triphosphate (ATP) production.[23–26] It has been shown that MB can act as an alternative electron carrier rerouting electrons from NADH to cytochrome *c*.[23–26] This process bypasses complex I/III blockage under pathological conditions and should theoretically ameliorate ROS production from the mitochondrial electron transport chain. Even during complex I inhibition by rotenone, MB can bypass electron transport chain blockage at complexes I and III, promoting respiration.[23–26]

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Methylene blue has been shown to rescue heart defects in a frataxin depleted drosophila model of FRDA.[27] Further analysis of MB derivatives in this model indicated that only compounds with electron carrier properties (redox active), were able to prevent heart dysfunction. Therefore, the ability of MB analogues to employ alternative mitochondrial electron transfer mechanisms may constitute a better therapeutic strategy for the treatment of FRDA.

In earlier studies involving methylene violet and other heterocyclic antioxidants as potential mitochondrial therapeutic agents, we found that the presence of a lipophilic substituent was essential to activity, presumably because interaction with mitochondrial respiratory complexes embedded in the inner mitochondrial membrane represent the locus of action.[26,28] Presently we describe seven lipophilic MB analogues, some of which are capable of increasing frataxin levels and mitochondrial biogenesis, in addition to improving mitochondrial function and supporting ATP synthesis in FRDA lymphocytes.

2. Results and discussion

2.1. Synthesis of the methylene blue analogues

In continuation of our previous research work on modifying parent compound MB to enhance biological activity, we have synthesized several new lipophilic MB derivatives (Fig. 1). The syntheses of MB analogues **1**–**7**, having long alkyl substituents on the phenothiazine redox core (Fig. 1), were carried out using a Wittig reaction as the key step. The synthesis of the lipophilic MB analogues began with the protection of the N atom at position 10 of commercially available 2-cyanophenothiazine (Scheme 1). Treatment of 2-cyanophenothiazine with NaH (60% in mineral oil) at 0 °C, followed by di-*tert*-butyl dicarbonate, afforded the desired product **8** in 72% yield. Reductive hydrolysis of protected cyanophenothiazine **8** by DIBAL-H and 2 N HCl afforded aldehyde **9** in 81% yield.[29] Treatment of **9** with each of six alkyltriphenylphosphonium bromides in the presence of 1 M NaHMDS afforded the corresponding intermediate alkenes (**10**–**15**) as *cis-trans* mixtures. The alkenes were subsequently reduced by catalytic hydrogenation over palladium-on-carbon to afford the corresponding alkanes (**16**–**21**) in good yields. In the final step, the Boc protecting group was removed using 10 equivalents of trifluoroacetic acid, then the intermediate was oxidized with iodine in CH_2Cl_2 followed by the subsequent addition of dimethylamine to afford analogues **1**–**6** (Scheme 1).[30] Alternatively, intermediate **21** was treated with morpholine to provide bis-morpholino derivative **7** in 28% yield (Scheme 2). Compound **7** was one of a few analogues containing more complex secondary amines at positions 3 and 7 of the phenothiazine nucleus. Of these, only compound 7 was evaluated fully, as noted below.

2.2. Biochemical and biological evaluation

2.2.1. Cytotoxicity

It has been reported that high doses of MB are detrimental and actually increase cytotoxicity and pathology.[26,31,32] A hormetic dose-response to MB has been described, suggesting that the beneficial effect of MB may be limited to a certain range of concentrations (<100 nM). This hormetic dose-response was attributed to the auto-oxidizing properties of MB.[33] Recently, we have reported new methylene violet analogues lacking cytotoxicity; these were found to be better antioxidants than the parent compounds.[26] In this regard, it seemed important to ensure that the prepared methylene blue analogues were not cytotoxic.

Accordingly, the MB analogues were evaluated for their cytotoxicity effects on FRDA lymphocytes grown in glucose free media (25 mM galactose). Since cells grown in galactose media as the only carbon source rely mostly on oxidative phosphorylation to produce their ATP, they become more sensitive to mitochondrial toxins than cells grown in high glucose media.[34,35] MB itself and analogues **1**–**3** exhibited significant cytotoxicity to FRDA lymphocytes at 2.5 μM concentration after a 48-h incubation (Fig. 2).

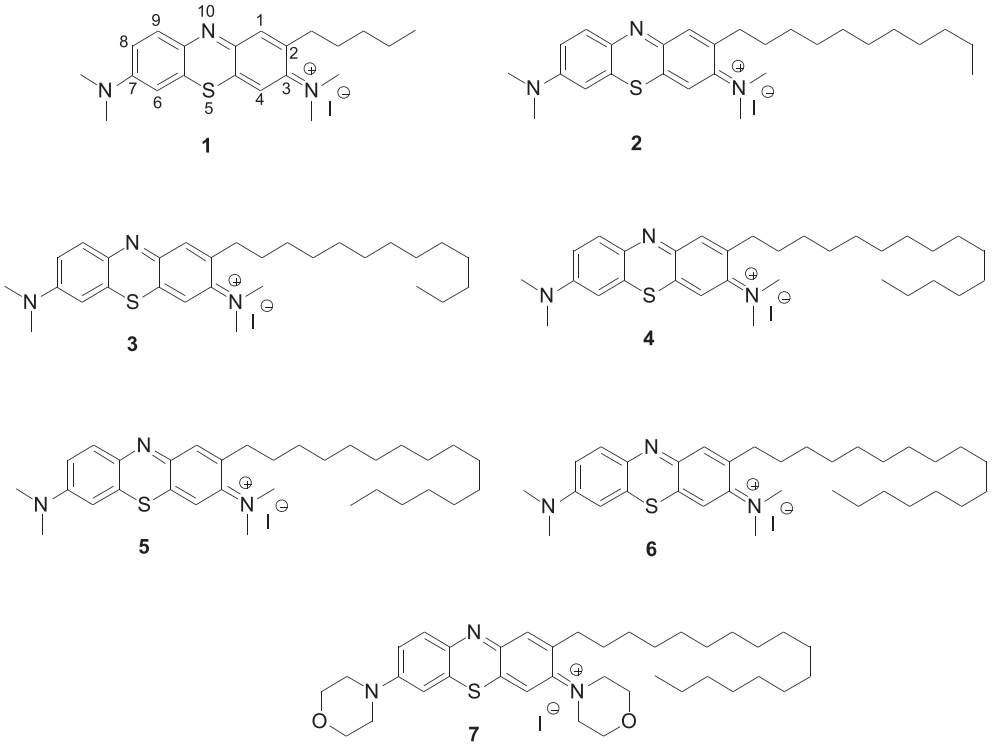

Fig. 1. Chemical structures of the newly synthesized methylene blue (MB) analogues.