Journal of Alzheimer's Disease 20 (2010) S439–S452
DOI 10.3233/JAD-2010-100414
IOS Press

Review

Protective Role of Methylene Blue in Alzheimer's Disease via Mitochondria and Cytochrome c Oxidase

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Accepted 15 April 2010

Abstract. The key cytopathologies in the brains of Alzheimer's disease (AD) patients include mitochondrial dysfunction and energy hypometabolism, which are likely caused by the accumulation of toxic species of amyloid-β (Aβ) peptides. This review discusses two potential approaches to delay the onset of AD. The first approach is use of diaminophenothiazines (e.g., methylene blue; MB) to prevent mitochondrial dysfunction and to attenuate energy hypometabolism. We have shown that MB increases heme synthesis, cytochrome c oxidase (complex IV), and mitochondrial respiration, which are impaired in AD brains. Consistently, MB is one of the most effective agents to delay senescence in normal human cells. A key action of MB appears to be enhancing mitochondrial function, which is achieved at nM concentrations. We propose that the cycling of MB between the reduced leucomethylene blue (MBH_2) and the oxidized (MB) forms may explain, in part, the mitochondria-protecting activities of MB. The second approach is use of naturally occurring osmolytes to prevent the formation of toxic forms of Aβ. Osmolytes (e.g., taurine, carnosine) are brain metabolites typically accumulated in tissues at relatively high concentrations following stress conditions. Osmolytes enhance thermodynamic stability of proteins by stabilizing natively-folded protein conformation, thus preventing aggregation, without perturbing other cellular processes. Experimental evidence suggests that the level of carnosine is significantly lower in AD patients. Osmolytes may inhibit the formation of Aβ species *in vivo*, thus preventing the formation of soluble oligomers. Osmolytes are efficient antioxidants that may also increase neural resistance to Aβ. The potential significance of combining MB and osmolytes to treat AD are discussed.

Keywords: Alzheimer's disease, amyloid-β, heme, methylene blue, mitochondria, osmolytes, oxidase, peroxidase

INTRODUCTION

Alzheimer's disease (AD) is a progressive dementia marked by failure to form new memories, thus interfering with the patient's normal daily activities. AD is the most common age-related dementia manifested by widespread progressive cognitive deterioration and impaired behavioral skills. The cost of caring for AD patients is staggering, and in the US exceeds $100 billion annually. Current drugs for AD can only remedy the symptoms with little or no effect on the basic mechanisms underlying the disease.

The most accepted view for the pathological mechanism of AD is the accumulation of small peptides known as amyloid-β (Aβ) in the brains of AD patients [1]. Aβ are the product of the proteolytic processing of a larger protein known as the amyloid-β protein precursor (AβPP). Three different proteases can cleave AβPP at three different locations. These proteases are designated as α-, β-, and γ-secretases. The cleavage of AβPP by β- and γ-secretases is consid-

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ISSN 1387-2877/10/$27.50

ered amyloidogenic cleavage because it produces intact Aβ peptide. If AβPP is cleaved by α-secretase, Aβ does not form and the cleavage is considered non-amyloidogenic. Interestingly, the sites of the proteolytic cleavage of AβPP by γ-secretase can vary. γ-secretase cleaves at several adjacent sites to create the c-terminal of Aβ. Thus, various Aβ species containing 39–43 amino acid residues are produced [2]. The cleavage of AβPP by γ-secrtetase increases with age and in response to specific risk and genetic mutations, increasing the production of the long species of Aβ [3–6]. We will collectively refer to all the forms as Aβ.

The long species of Aβ (A$\beta_{42,43}$) are strongly amyloidogenic and aggregate more readily than the short form of Aβ (A$\beta_{39,40}$); thus, they may provide the nucleation seed for the formation of Aβ_{40} aggregates, which ultimately are the building blocks for the senile plaques formation [7]. Senile plaques are insoluble extracellular Aβ deposits, in which Aβ forms insoluble fibrils. Senile plaques are one of the neuropathological hallmarks of AD mainly made from Aβ peptides; however, other proteins can also be detected in senile plaques but at very low abundance [7].

In addition to the oligomeric and fibril forms of Aβ, studies have shown that AD is also marked by neurofibrillary tangles and other key cytopathologies (reviewed in [8]). Some of the key cytopathologies in AD include decline in cytochrome c oxidase (complex IV), mitochondrial dysfunction, and energy hypometabolism. The molecular link between Aβ and the key cytopathologies of AD is still debated [9–12]. In the following sections, we will discuss each of these elements, and new views into Aβ neurotoxicity and delaying the onset of AD.

AMYLOID-β, OLIGOMERS, AND FIBRILS

Because of the natural tendency of Aβ to form oligomers, aggregates, and senile plaques, AD is defined as a disorder of protein aggregation and misfolding. The factors that trigger protein misfolding *in vivo* and with age are not yet clear. However, genetic polymorphism, as well as environmental and lifestyle factors may be important risk factors for Aβ peptide accumulation and aggregation [3–6].

Several lines of research show that an increase in soluble dimeric and oligomeric forms of Aβ, which precede the appearance of senile plaques [13–15], correlate with early cognitive impairment in AD [16,17]. Furthermore, experiments using cultivated cells, transgenic mouse models for AD, and human brain autopsy tissue suggest that soluble Aβ oligomers are likely the primary toxic forms of Aβ. Aβ oligomers are also elevated in human-AβPP transgenic mice for Aβ accumulation and AD [18] and most significantly, in AD brain [19]. Thus, preventing the aggregation of Aβ peptides is a plausible approach to delay AD. Interestingly, experimental evidence also shows that Aβ can be found not only at the extracellular milieu, but also inside the cell and even in the mitochondria [20, 21], stressing the view that intracellular Aβ as well as extracellular Aβ could also be neurotoxic [22]. This is a major departure from the older view that senile plaques are the main neurotoxic factor in AD. Senile plaques may contribute to AD by slowly shedding to their surrounding the soluble oligomeric form of Aβ. Thus, methods for stabilizing senile plaques may delay the onset of AD by lowering the oligomeric forms of Aβ. In this article, we propose a pharmacological approach to enhance mitochondrial function and prevent the formation of Aβ toxic oligomers.

It is not clear which of the key cellular compartments (i.e., cytosolic, mitochondria, synapses) is the primary target of soluble oligomeric Aβ toxicity. Mitochondria appears to be a target for Aβ toxicity as we will discuss below. Synaptic dysfunction is also proposed to be the primary neural malfunction that disturbs cortical neural circuits and causes short-term memory impairment in AD patients [23]. Synaptic dysfunction is suggested to precede neural death and appears to be the target of Aβ neurotoxicity. This hypothesis emerged from experiments showing that Aβ oligomers inhibit long-term potentiation (LTP), a classic experimental paradigm for memory and synaptic plasticity [24,25]. Impairment of energy metabolism by intraneuronal Aβ inhibition of key mitochondrial functions may also contribute to synaptic dysfunction in AD. Thus, preventing mitochondrial dysfunction is an additional plausible approach to delay AD. Our goal is to identify the primary metabolic pathway that is specifically targeted by excess Aβ [26].

COMPLEX IV, MITOCHONDRIA, AND ENERGY METABOLISM

The key cytopathologies of AD include decline in cytochrome c oxidase (complex IV), mitochondrial dysfunction, energy hypometabolism, abnormal iron homeostasis, oxidative stress, dimerization of AβPP, and synaptic dysfunction [27–31]. Selective decline

in mitochondrial complex IV seems to contribute to mitochondrial dysfunction, energy hypometabolism, and oxidative stress (e.g., increased production of H_2O_2) [32–34]. The molecular link of Aβ to these cytopathologies is not clear. Soluble oligomeric forms and intraneuronal Aβ could be critical for understanding the mechanism of neurotoxicity of Aβ. We have recently proposed a new model for Aβ toxicity in which strong binding of Aβ with heme is key for the neurotoxicity of Aβ. Aβ binding with heme leads to the depletion of regulatory heme and the decline in complex IV activity among other effects [35,36]. Thus, heme metabolism could be the primary metabolic pathway that is specifically targeted by excess Aβ.

Mitochondria appear to be a direct target for Aβ. Aβ peptides can be detected in the mitochondria [37–39]. Furthermore, this view was strengthened because emerging evidence point to that Aβ may exert its toxicity, in part, by interfering with specific mitochondrial structures and functions. For example, Aβ may increase the production of nitric oxide (NO), which triggers mitochondrial fission (fragmentation) leading to mitochondrial damage and impaired energy metabolism [40]. S-nitrosylation of dynamin-related protein 1 (Drp1) is a possible candidate for this effect of NO. Nitrosylation of Drp1 is increased in AD brains [40]. In the same line with these findings, Aβ disturbs the normal balance in mitochondrial fission and fusion that leads to smaller mitochondria and abnormal intracellular distribution [41]. This, abnormal biology of the mitochondria may contribute to mitochondrial and neuronal dysfunction seen in AD [41]. The effect of Aβ on the permeability transition pore has also been reported. Aβ's toxicity to mitochondria seems to require Aβ binding with cyclophilin D, a key component of the formation of permeability transition pore and thus regulating cell death [42].

Several studies have demonstrated changes to the TCA cycle, which is an important metabolic pathway in mitochondria, in addition to electron transport and complex IV. Researchers have demonstrated a 30–40% decrease in complex IV activity [43,44] in addition to α-ketoglutarate dehydrogenase (αKGDH) [45]. A more comprehensive screening of the activities of the TCA cycle enzymes in AD revealed that some decreased (e.g., PDH, αKGDH, and ICDH), others increased (e.g., SDH and MDH), while the four other enzymes did not change (e.g., aconitase) [46]. These changes presumably would result in a decline in succinyl-CoA, the intermediate of the TCA cycle that is produced by α KGDH and consumed by the subsequent reactions of SDH and MDH. Succinyl-CoA is the precursor for heme synthesis [47]. Thus, decline in succinyl-CoA would be expected to synergize with the consequences of Aβ complexing with heme and exacerbate the depletion of regulatory heme [26,48]. In addition to the biochemical changes, the mitochondria from the brain of AD patients exhibits substantial structural changes that included abnormal cristae, accumulation of osmiophilic material, and smaller size compared to normal controls [21,49]. The decline in complex IV, which occurs in heme deficient cells, leads to similar structural consequences on mitochondria (unpublished observations).

AMYLOID-β PEPTIDE BINDS WITH SPECIFIC METABOLITES

Several studies have demonstrated an abnormal interaction of Aβ with key brain metabolites. Aβ can bind iron, zinc, copper, cholesterol, mitochondrial protein import machinery, HrtA2 protease, ABAD, and heme [20,26,37,50–52]. The biochemical and metabolic consequences of the interaction of Aβ and these metabolites are under investigation, especially their relation to landmark cytopathologies of AD, such as the decline in complex IV, loss of iron homeostasis, and energy hypometabolism.

Several lines of experimental evidence provided support that heme metabolism may be a specific metabolic pathway that is targeted by Aβ peptides [26,53–55]. We recently showed a specific heme-binding motif in human Aβ peptides [26]. These findings led to the proposal that depletion of regulatory heme and the formation of Aβ-heme peroxidase are key factors in energy hypometabolism, mitochondrial dysfunction, oxidative stress, loss of complex IV, and impaired iron homeostasis seen in the brains of AD patients (reviewed in [8]).

Heme-binding with Aβ could also explain why humans, but not rodents, develop AD-like neuropathology. Compared to human Aβ (huAβ), amino acids Arg5, Tyr10, and His13 are replaced with Gly5, Phe10, and Arg13, respectively, in rodent Aβ (roAβ) (reviewed in [8]). The phylogenic variation in the amino acid sequences of Aβ led to differential heme-binding between huAβ and roAβ. huAβ binds heme with high affinity ($K_d = 1.4 \times 10^{-7}$ M), while the K_d for roAβ was ten-fold less. These findings stress the significance of the three amino acids: Arg5, Tyr10, and His13 in heme-binding by huAβ. The amino acids Arg, Tyr, and His are known to also participate in heme-binding

in heme-proteins and peroxidases [56–58], which provide biochemical support for the observed peroxidase activity of huAβ-heme.

huAβ is likely to bind with "regulatory" heme *in vivo*. The intracellular concentration of "regulatory" heme is estimated to be between 0.3 and 1.5×10^{-7} M [59], indicating that huAβ is likely to form an Aβ-heme complex in the brains of AD patients. Consistently, heme has recently been found to colocalize with the senile plaques in AD brain [53]. RoAβ, on the other hand, is far less likely to bind with heme *in vivo* since the K_d is higher than the concentration of regulatory heme.

Heme is the major functional form of iron and is responsible for the metabolic integrity of complex IV, the mitochondria, and the regulation of key gene regulation systems. Regulatory heme provides heme for all the biochemical pathways that depend on heme (e.g., assembly of heme-proteins, gene regulation, processing of microRNA) [7,60,61]. Thus, saving regulatory heme by lowering Aβ oligomeric forms and enhancing heme synthesis should improve neuronal energy metabolism.

CURRENT THERAPEUTIC APPROACHES AND THE CHALLENGE

Despite the intensive research on AD and the depth to which we understand the cell biology and biochemistry of Aβ (and to some extent AβPP), a therapy or prevention strategy for AD remains elusive [62]. The absence of a definite molecular mechanism to explain the neurotoxicity of Aβ is a key obstacle for the development of effective therapies to treat AD. Drugs currently available to treat AD patients (e.g., blocking NMDA glutamate receptors, acetylcholine esterase inhibitors) are targeted at treating only the symptoms of AD and their efficacy is also limited. The efficiency of these drugs progressively declines as the disease progresses, indicating that they do not alter the underlying neurodegenerative mechanism of Aβ. Furthermore, these drugs also seem to be ineffective in some patients. Thus, there is an urgent need for a new generation of drugs to prevent or delay the onset of AD.

A reliable and accurate diagnostic biomarker for AD is also lacking. Progress is being made on this front by relying on imaging techniques, abnormal brain structures, and Aβ deposits [63], all of which specify the clinical aspects of the disease itself. Thus, in order to benefit from the full potential of imaging techniques, they must be accompanied by a cure for AD, which currently is not available. The ultimate biomarker would preferably be a blood- or CSF-borne metabolite that indicates the risk for AD in advance of the clinical signs. Furthermore, as is the case for imaging techniques, for such a biomarker to be utilized to its full potential, a prevention method is needed. The established mitochondrial dysfunction in AD creates an additional therapeutic route to explore possibilities to enhance mitochondrial function and delay the onset of AD. Below, we discuss a new approach to prevent the decline in mitochondrial complex IV, enhance heme production, and prevent the formation of the toxic species of Aβ peptide.

OLIGMOERS AND MITOCHONDRIA AS TARGETS FOR DELAYING AD

The brain is particularly sensitive to mitochondrial dysfunction, the resulting oxidative stress, and impaired energy metabolism [64–66]. Energy deficiency can result in synaptic dysfunction and neurodegeneration of the hippocampus and cortical regions of the brain [67]. A promising approach to improve the brain's energy metabolism has been shown using intranasal delivery [68] to elevate the level of insulin in the brain [69], which may improve glucose metabolism. An additional approach to improve energy metabolism is to enhance the activity of the mitochondria in the brain. Both the glucose metabolism and mitochondria are interconnected, thus an approach to improve both aspects of energy metabolism might be more effective.

Insoluble protein aggregates and misfolded protein deposits such as Aβ oligomers, senile plaques, phosphorylated tau (p-tau), α-synuclein, and polyglutamine are neuropathologies in many neurodegenerative diseases. Aβ oligomers in AD usually progress to form large insoluble plaques, which exacerbate neurodegeneration, indicating that the human brain has a limited capacity of disposal of Aβ aggregates. Decline in proteosome activity with age may contribute to the accumulation of the misfolded protein deposits [70]. The aging brain also exhibits limited capacity of self-repair (e.g., limited neurogenesis).

Our knowledge of the kinetics of the formation of Aβ oligomers *in vivo* as well as the mechanism by which they interfere with the function of distinctive set of neurons is limited. We propose that intermediate species in the cascade of protein misfolding may become highly toxic by interfering with basic metabolic activity of the brain. We demonstrated that the binding

of Aβ (and the soluble oligomers of Aβ) with heme results in sequestration of regulatory heme leading to impaired cellular metabolism. Therefore, preventative approaches to treating AD could be targeted at blocking the formation of Aβ oligomers and enhancing the synthesis of heme and complex IV, in addition to the use of antioxidants.

Preventing aggregation and oligmoerization of amyloid-β with osmolytes

Proteins need to maintain their functionally active conformation(s) under physiological conditions [71]. Thus, under severe physical and chemical stress conditions, proteins must counter-balance any significant perturbations in the thermodynamic conditions to avoid significant changes in their conformations [72–75]. Proteins failing to adapt to such conditions may result in misfolding/aggregation, which lead to a partial or complete loss of their functional activity [76,77]. In order to adapt to such perturbations, tissues create certain mechanisms such as accumulation of small organic solutes, also known as "osmolytes" [72,73,78]. These osmolytes maintain stability and folding of proteins without perturbing other cellular processes, and are typically accumulated intracellular at relatively high concentrations [74,75,77,79–81]. It is a well known fact that once proteins are synthesized, the highly disordered unfolded state passes through well-defined partially structured transition states to fully folded forms, aided by molecular chaperones that deter aggregation of incompletely folded species [82]. Under certain conditions, protein unfolds, at least partially, and becomes prone to aggregation [83–85]. This may result in the formation of fibrils and other possible aggregates that accumulate in tissue [86–91]. It is likely that small aggregates, as well as the highly organized fibrils and plaques, can give rise to pathological conditions, a common feature among many neurodegenerative diseases, including AD [81,92].

There are a number of well-known naturally occurring osmolytes, which fall into three chemical classes: methylamines (trimethylamine-N-oxide, Choline-O-sulphate, and sarcosine), polyols (sorbitol, glycerol, sucrose, and trehalose), and certain amino acids (glycine, taurine, proline, and betaine). Some of these osmolytes are shown in Scheme 3. Each of these interacts with the peptide backbone and amino acid sidechains [93]. The potency of the osmolyte to promote protein folding and solubility is determined by the balance of these effects. There are several studies to support the view that the powerful solvophobic effects of osmolytes on the peptide backbone dominate, such that the relative Gibbs free energy (ΔG) of the unfolded state is less favorable than that of the folded state.

The presence of several osmolytes inside cells raises questions about their roles in protecting intracellular macromolecules under pathological conditions. Since the protection provided by an osmolyte does not depend on specific chemical interactions with the macromolecules, in principle, any of the osmolytes should be capable of replacing each other, depending upon either endogenous or exogenous availability of particular osmolyte(s) [94]. Since the role of protein backbone is critical in determining thermodynamic stability and folding of proteins in osmolyte solutions [95–99], designing these small molecules (osmolytes) appears to be an excellent strategy and could be a critical step in preventing various critical proteins from misfolding or aggregating [6]. This may have far reaching consequences in understanding and preventing several deleterious diseases that are caused by protein misfolding/aggregation [100,101]. Since organic osmolytes are naturally occurring molecules, they may have potential therapeutic applications without concerns of major toxic side effects [102].

It has been shown that oral administration of an osmolyte (trehalose) can significantly inhibit polyglutamine-mediated protein aggregation in the transgenic mouse model of Huntington's disease (a neurodegenerative disease) and subsequently increase life span [98]. Another study has demonstrated that osmolytes can be used to fold androgen receptor containing elongated polyglutamine chain length, which leads to formation of protein aggregates and is responsible for a neurodegenerative disease [100]. Because of their protein stabilizing capabilities, osmolytes have been the focus of several studies related to neurodegenerative diseases in which the pathogenesis is associated with the misfolding of specific proteins [103,104]. These diseases include AD, Huntington's disease, and muscular dystrophy. In these pathological conditions, specific misfolded aggregate-prone proteins are resistant to the normal cellular processes. Insoluble aggregates are found to correlate with the progression of these diseases. Osmolytes are thought to work by interfering with the production and/or enhancing destruction of these toxic entities. Thus, osmolytes have become an attractive molecule for study in neurodegenerative diseases characterized by protein misfolding and aggregate pathology.

Osmolytes have also been found beneficial in transgenic model systems for oculopharyngeal muscular

dystrophy, an autosomal dominant disease [105]. Despite of the fact that, in AD, toxicity is increasingly linked to the formation of oligomeric forms of Aβ peptides, AD progression is correlated with increasing aggregate formation of Aβ, like many other neurodegenerative diseases [103]. In AD, Aβ_{40} is the most prominent peptide and Aβ_{42} is more toxic. It has been reported that at the physiological concentration, Aβ_{40} peptide incubated in the presence of an osmolyte, trehalose inhibits aggregation of this peptide in a dose-dependent manner [106]. This osmolyte-mediated inhibition of Aβ_{40} peptide aggregation correlates with its toxic effects in neuronal cell system [106]. Thus, osmolytes may have potential as part of a therapeutic strategy for treating AD, because of their effect on Aβ oligomers. Osmolytes may also help stabilize the senile plaques, preventing the shedding of Aβ oligomers. Osmolytes are also known to function as antioxidants [107,108], and their level seems to decline in AD patients [109]. These studies suggest that naturally occurring osmolytes may have a protective effect in promoting brain health, including AD. Thus, there is potential for both prevention and treatment of neurodegenerative diseases [110,111]. The prospect of using natural osmolytes as a therapeutic tool for AD and several other neurodegenerative diseases appears to be quite exciting without fear of major side effects. However, while these studies appear to be quite promising, more studies are needed to validate their effectiveness as a potential therapeutic target. Protein aggregation/misfolding constitutes a hallmark of neurodegenerative diseases including AD, as understanding the proper mechanism of action of osmolytes in these pathological conditions can have far reaching consequences in developing better therapeutic tools for the prevention and/or management of such diseases.

Preventing the decline in complex IV with methylene blue

Mitochondrial dysfunction in AD is marked by a decline in complex IV, decline in other TCA cycle enzymes, and mutations to mtDNA. Impairment to mitochondrial complex IV increases the production of free radicals and oxidants, such as hydrogen peroxide (H_2O_2). Energy deficiency is an additional serious result of impaired mitochondrial function. Furthermore, the role of mitochondria in cellular senescence and aging has also been demonstrated, which may contribute to neural dysfunction with age. Mitochondrial medicine is an emerging field of research directed at finding therapeutic strategies to enhance mitochondrial function to combat aging and neurodegenerative disorders. This direction of research led to the discovery of several agents that can delay aging in human primary cells in culture or in other research models (reviewed in [112]). These agents are proposed to target basic mechanisms of aging, including mitochondria. The efficacy of these agents to delay aging *in vivo* is under investigation [113]. Thus, mitochondria-protecting agents may also be potential drugs to prevent or delay age-related neurodegeneration (e.g., AD).

We recently showed that MB is unusually effective in counteracting some key basic mechanisms of mitochondrial dysfunction [114]. MB increases mitochondrial complex IV by 30%, enhances cellular oxygen consumption by 37–70%, increases heme synthesis, and reverses premature senescence caused by H_2O_2 or cadmium. We showed that MB delays senescence human lung fibroblasts at nM levels. Enhancing mitochondrial function could contribute to the antisenescence activity of MB. We also found that the activity of complex IV in brain of old mice was doubled upon treatment with MB for three months [115].

The ability of MB to cross the blood brain barrier also has been demonstrated by previous studies [116]. We found that when MB is administered in drinking water to old mice for three months, the brain concentration does not exceed 120 nM [115]. This concentration of MB is consistent with the 100 nM required for optimal effect on mitochondria *in vitro* [114].

THE SIGNIFICANCE OF INDUCING COMPLEX IV AND HEME SYNTHESIS WITH MB TO THE PREVENTION OF AD

MB is the first chemical to be capable of inducing mitochondrial respiratory complex. MB also enhances the capacity of the cells to synthesize heme and delay cellular senescence. Thus, we propose a new medical use for MB; an agent that may postpone the onset of AD by delaying the decline in complex IV and increasing heme synthesis. If we assume that a critical threshold of complex IV and heme level determines the age of onset of synaptic dysfunction and energy hypometabolism in AD, then MB may increase the brain's reserve of both complex IV and the capacity to synthesize heme (Fig. 1).

Increasing the activity of complex IV above normal levels is intriguing. Normally, complex IV is found in $\approx$5 fold excess over the other electron transport com-

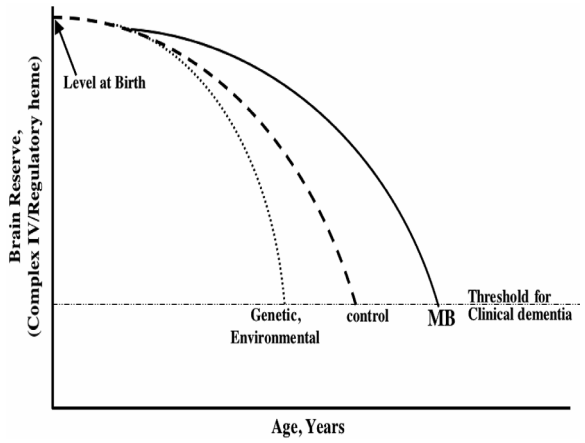


Fig. 1. The trajectory for the effect of MB on the age-dependent decline in complex IV or regulatory heme. This is hypothetical scheme presenting the trajectory with age (X-axis) for brain reserve at birth (Y-axis) of complex IV (or heme). Brain reserve declines with age until it reaches a critical threshold after which clinical signs become evident. We anticipate that MB may increase the brain reserve of complex IV and heme, thus extending the time to clinical threshold leading to a delay in the onset of clinical dementia (in this case AD). An extreme case is depicted when genetic mutation or exposure to environmental factor, which may accelerate the decline in complex IV and heme.

plexes of the mitochondria [117], which may indicate the physiological significance of excess complex IV. However, there is no definitive answer as yet to the physiologic significance of the excess in complex IV, though minor decline in complex IV leads to disorders of energy metabolism and mitochondrial dysfunction. A decrease in complex IV activity causes cytotoxicity, leads to specific pathologies [118], increases oxidants production, and decreases the energy charge of the mitochondria [119,120]. On the other hand, the effect of an increase in complex IV on oxidants formation and energy metabolism is not known. Based on our *in vitro* and *in vivo* results, we anticipate that elevated levels of complex IV would improve mitochondrial function.

Energy production by mitochondria relies on four electron transport complexes (also known as the electron transport chain, ETC), which are complex I, II, III, and IV. Electron transfer through each one of the complexes starts at complex I, which catalyzes a two-electron oxidation of NADH, and continues until complex IV forms water. Complex IV consumes more than 95% of the O_2 that reaches the cell. The production of H_2O_2 and other oxidants appears to be enhanced by the stalling of electrons upstream of complex IV (at complexes I and III) [32,121–123]. Complexes I and III are two sites responsible for the production of free radicals by non-specific transfer of electron to O_2. Thus, excess complex IV may play a key role in decreasing the steady-state concentration of intracellular O_2 and, as a result, lowering the production of oxidants by the mitochondria. Furthermore, a high level of complex IV correlates with the metabolic activity of the cell and with cognitive performance.

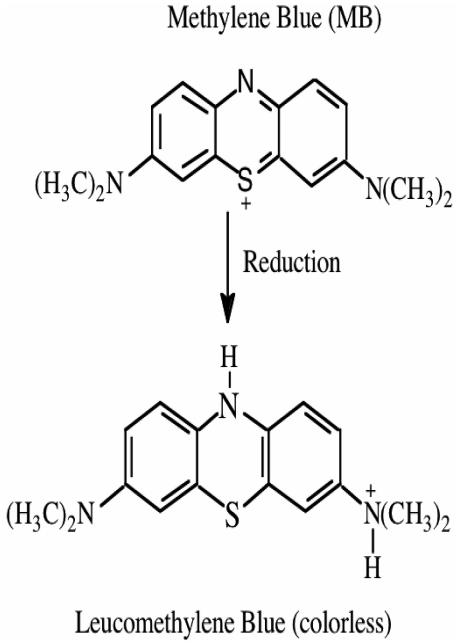
A decline in H_2O_2 production upon induction of complex IV limits the catalytic activity of huAβ-heme perxidase [48], which requires H_2O_2 as a co-substrate. Furthermore, enhancing heme synthesis should provide neural cells with better reserve and capacity to provide regulatory heme, thus delaying the onset of the consequences of sequestration of heme by huAβ. These effects of MB on cell metabolism and mitochondrial complex IV in particular suggest that MB may delay the onset of AD (Fig. 1).

Aggregation of p-tau has been prevented at $\approx$3 μM MB in test tube [124]. Furthermore, MB at a concentration higher than 15 μM has been shown to inhibit Aβ oligomerization by promoting fibrillization [125]. The concentration of MB used are very high and may lead to neurotoxicity [114,126]. MB exhibits cellular toxicity at μM range of concentration and may harm the nerve cells. Our findings show that MB is effective at improving mitochondrial function at nM range of concentrations, which is consistent with the intra brain concentration that can be achieved upon chronic treatment with MB [115].

MB has a promising potential to delay the onset of AD [114,127] since it also has the added benefit of being known for human use; it has a very long-standing, extensive history of medical uses [128]. Thus, MB is a drug with an extended medical and safety record in humans, and FDA approval for its testing in clinical trials in connotation to aging and age-related disorders may not be denied on safety grounds.

PROPERTIES OF METHYLENE BLUE

Methylene blue (3,7 Bis-dimethylamino-phenazathionium) is known as a redox indicator with a redox potential of 11 mV. The low redox potential allows MB to cycle readily between oxidized and reduced forms by various redox centers such as those present in mitochondria (Scheme 1). Several NAD(P)H-dependent dehydrogenases also reduce MB to form the colorless leucomethylene blue (MBH_2). MBH_2 can be reoxidized to MB by O_2 in the absence of suitable electron acceptors such as cytochrome c [129] or other hemeproteins [130].



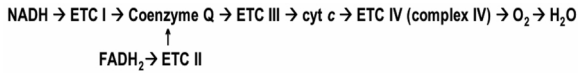
Scheme 1: The oxidized and reduced structures of methylene blue.

MB is soluble in both water and organic solvents; the lipid solubility of MBH_2 is higher than that of MB. Electron delocalization in MB results in a partial positive charge being located on both nitrogen and sulfur atoms, which may increase the permeability of MB through membranes. Thus, MBH_2 and MB can enter the mitochondria and other intracellular compartments such as lysosomes [131].

When exposed to high intensity of UV light, MB causes oxidative damage to isolated DNA. No such toxicity has been shown in humans [132], presumably because it requires high exposure to UV and most MB *in vivo* is in the reduced form of MBH_2, which does not have photodynamic activity [133].

Clinical uses of methylene blue

MB has been in clinical use for decades to treat a variety of pathological conditions and diseases. MB is readily absorbed and quickly distributed to various organs including the brain [134]. One of the most common uses of MB is the chronic treatment of congenital methemoglobinemia, which is due to methemoglobin reductase deficiency. MB is also used to treat methemoglobinemia caused by cyanide, CO, or nitrate poisoning [135]. Recent clinical uses for MB include preventing the side effects of chemotherapy (e.g., ifosfamide-induced encaphelopathy [136]) and preventing hypotension in septic shock [137]. MB is



Scheme 2: The electron transport chain (ETC) of the mitochondria. The four complexes are: complex I (ETC I), complex II (ETC II), complex III (ETC III), and complex IV (ETC IV) in addition to ATP synthase (i.e., complex V). The electron transfer through each one of the ETC starts at ETC I, which catalyzes two electrons oxidation of NADH and continues until water is formed on ETC IV. Coenzyme Q serves as low molecular weight electron carrier from ETCs I and II to III. Cytochrome c (cyt c) serves as electron carrier from ETC III to ETC IV.

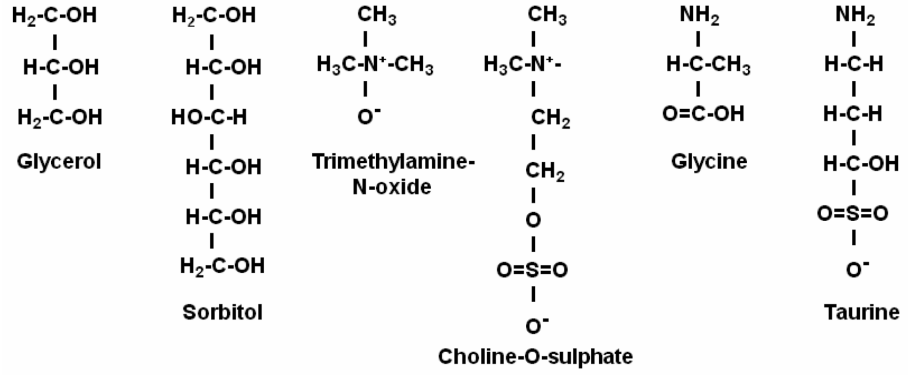
also used in the treatment of some psychiatric disorders because of its anxiolytic and antidepressant properties [138,139]. The typical dose of MB in clinical uses ranges between 1-2 mg/kg/day; however, MB can also be repeatedly administered at this dose up to 6 times over 24 h [135]. High non-therapeutic doses ($>$ 7.5 mg/Kg) of MB cause the formation of Heinz bodies in erythrocytes in humans [140] and impairs hematological parameters in rodents [141].

Research into additional medical uses of MB is in progress. MB has been shown to protect against cyclosporine injury to the kidney [142] and streptozotocin injury to the pancreas [143]. MB also protects from ischemic-reperfusion injury [144], radiation [145], and enhances β-oxidation of long chain fatty acids [146].

Administering MB *in vivo* benefits the central nervous system: MB improves the cognitive function in rats and increased the activity of cytochrome c oxidase (complex IV) in the brain by 25% (after single high dose) [147,148]. Previous findings show that MB inhibits the activity of monoamine oxidase in the brain, which may lead to an increase in the concentration of dopamine [149].

Proposed mechanisms of actions of methylene blue in current clinical applications

The mechanism(s) of the diverse biological actions of MB described above are not clear. MB has been proposed to act by inhibiting the NO-activating soluble guanylate cyclase (sGC) [150] (though the basal activity of sGC is not affected); inhibiting nitric oxide synthase (NOS) [126]; inhibiting MAO [149]; and acting as an antioxidant precursor [130,151]. The effects of MB were measured at concentrations greater than 10 μM. However, recent studies showed the effects of MB are inconsistent with the above proposed mechanisms [114,152]. These discrepancies may be because MB exhibits different effects at different concentrations ($>$ 10 μM versus $<$ 1 μM). Thus, these mechanisms



Scheme 3: Examples of some well-known osmolytes.

may explain some, but not all of the biological actions of MB, we observed when using MB at nM concentrations [114].

Using our experimental findings in conjunction with previous studies on MB, we proposed for the first time a molecular mechanism explaining, in part, the effect of MB on mitochondria [114]. Briefly, MB can readily cycle between the oxidized (MB) and the reduced (MBH_2) forms [153]. NAD(P)H-dependent dehydrogenases (e.g., NADH-dehydrogenase of complex I) can reduce artificial electron acceptors such as MB to MBH_2 or O_2 to superoxide radical [122]. Thus, we proposed that MBH_2 and MB serve as electron carriers between several dehydrogenases and heme-proteins (e.g., cytochrome c). In turn, complex IV recycles the reduced cytochrome c (Scheme 2). If this cycling of MB occurs within the mitochondrial ETC, we predicted an inhibition of the production of superoxide radical by MB by competing with O_2 at the site of free radical production at NADH-dehydrogenase of complex I [122]. NADH-dehydrogenase of complex I faces the matrix, thus part of the MBH_2 will be formed in the mitochondrial matrix in addition to the cytosol. The hydrophobicity of MBH_2 increases upon reduction, thus increasing the chances that MBH_2 crosses the mitochondrial inner membrane and reaches cytochrome c and complex IV. This mechanism has previously been explained in more detail [114].

Intracellular MB is likely to cycle between the oxidized (MB) and the reduced (MBH_2) forms. Although numerous NAD(P)H-dependent enzymes can reduce MB to MBH_2, cytochrome c in the mitochondria and methemoglobin (in the red blood cells) are the only heme proteins reported to reoxidize MBH_2 to MB [129, 130]. Cytochrome c is an electron carrier from complex III (the natural enzyme that reduces cytochrome c) to complex IV (the natural enzyme that oxidizes cytochrome c). Thus, MBH_2 may increase the rate of the reduction of cytochrome c over and above the normal enzymatic reduction by complex III. Complex IV catalyzes the electron transfer from the reduced cytochrome c to O_2 to form H_2O. Increased reduction of cytochrome c by MBH_2 [129,130] could explain, in part, the increase in complex IV that occurs in the presence of 100 nM MB [114]. Cycling of MB between oxidized and reduced forms may block oxidant production by mitochondria. Mitochondrial dysfunction and oxidative stress are thought to be key aberrations that lead to cellular senescence, aging, and AD.

Adequate assembly and activity of complex IV depends on heme-a [154], which exists only in complex IV. Thus, the increase in the level of complex IV requires an increase in heme, which is the precursor for heme-a. The increase in the rate in heme synthesis we observed at 100 nM MB, could provide cells with the heme-a to support the assembly of complex IV.

Complexes I and III of the mitochondria, which are upstream to complex IV, are implicated in the production of free radicals by non-specific transfer of electrons to O_2. Thus, stalling electrons at ETCs upstream of complex IV enhances production of oxygen free radicals. Close to 95% of the intracellular O_2 is consumed by complex IV. Consistent with the increase in complex IV by MB, we found an increase in O_2 consumption in cells maintained in 100 nM MB. Thus, possible advantages of high level of complex IV is minimizing "electrons dwelling" at complexes I and III and increasing the conversion of the O_2 to water. Both effects of complex IV act to decrease oxidant production (e.g., H_2O_2) [32]. Thus, elevated complex IV induced by MB may lower the oxidative stress and oxidative damage. High levels of complex IV also increase mitochondrial activity, which would improve energy status.

SUMMARY

We proposed two different approaches to prevent or delay the onset of AD. The first is directed at enhancing mitochondrial activity using MB to increase the level of complex IV (Fig. 1). Successful merger of treatment with MB and intranasal delivery of insulin to the brain [68] may prove valuable for AD patients. Energy deficiency in AD may be contributed by impaired insulin (glucose) metabolism and mitochondrial function. Thus, concentrating on single impairment at the time would not be enough to resolve the energy hypometabolism in AD. Glucose metabolism depends on adequately functioning mitochondria and vice versa. Since both glucose and mitochondrial metabolism are interconnected, it might be more beneficial for AD patients to develop a therapeutic approach that resolves (or delays) both impairments. MB exerts its effect at very low (nM) concentration, which in conjunction with its safety record in humans, further minimizes any risk of side effects of chronic exposure to MB. The second approach is directed at preventing the aggregation of Aβ by using osmolytes, natural metabolites synthesized in the brain. Preventing the aggregation of Aβ may enhance their proteolytic removal and decrease the risk of their interference with heme and mitochondrial metabolism. MB can also induce heme synthesis, thus, when combined with osmolytes, may assist in preventing heme deficiency. We propose MB and osmolytes could help delay the onset of AD by preventing Aβ oligomers formation, enhancing mitochondrial function, and attenuating heme deficiency.

ACKNOWLEDGMENTS

Study was supported in part by Ames foundation and AFAR (HA) and NIH (RK). We are grateful for Prof. William H. Frey II, Jennifer Cawley, and Doaa Atamna for reading the manuscript.

Authors' disclosures available online (http://www.j-alz.com/disclosures/view.php?id=415).

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