Cellular and Molecular Actions of Methylene Blue in the Nervous System

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Abstract

Methylene Blue (MB), following its introduction to biology in the 19th century by Ehrlich, has found uses in various areas of medicine and biology. At present, MB is the first line of treatment in methemoglobinemias, is used frequently in the treatment of ifosfamide-induced encephalopathy, and is routinely employed as a diagnostic tool in surgical procedures. Furthermore, recent studies suggest that MB has beneficial effects in Alzheimer's disease and memory improvement. Although the modulation of the cGMP pathway is considered the most significant effect of MB, mediating its pharmacological actions, recent studies indicate that it has multiple cellular and molecular targets. In the majority of cases, biological effects and clinical applications of MB are dictated by its unique physicochemical properties including its planar structure, redox chemistry, ionic charges, and light spectrum characteristics. In this review article, these physicochemical features and the actions of MB on multiple cellular and molecular targets are discussed with regard to their relevance to the nervous system.

1. Introduction

In the late nineteenth century, the increasing demand for dyes in the prospering textile industry brought rapid advancements in the research of synthetic dyes. One of the most obvious results of this progress was the introduction of a vast number of colors into the everyday life of European cities (Streba et al., 2007). The synthesis of the first aniline-based dyes, such as mauve by William Perkin in 1856, led to an increase in their popularity and encouraged research on the use of aniline derivatives as dye precursors. In 1876, Methylene Blue (MB) was synthesized by Heinrich Caro of Badische Anilin und Soda Fabrik (BASF) as an aniline-based dye for cotton staining. A year later, BASF was awarded Germany's first dye patent (Caro, 1877). Although MB (Swiss blue, aniline violet, methylthionine hydrochloride, tetramethylthione hydrochloride) failed to live up to the standards of the textile industry, scientists such as Robert Koch and Paul Ehrlich were quick to realize that it was not only possible to stain different cellular structures with different dyes, but also to *stain* and *inactivate* microbial species *selectively*. In turn, this discovery led to the testing of these dyes against tropical diseases, and the first candidate to be administered to humans was MB, which Ehrlich showed in 1891 to be effective in the treatment of malaria (Guttmann and Ehrlich, 1891). Thus, MB was the first synthetic compound ever used as an

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antiseptic in clinical therapy and the first antiseptic dye to be used therapeutically. In fact, the use of MB and its derivatives was widespread before the advent of sulfonamides and penicillin in chemotherapy (for a review Wainwright and Crossley, 2002). MB has also been a lead compound in drug research against various bacterial and viral infections (Wainwright and Crossley, 2002) and cancer (Wainwright and Crossley, 2002; Wainwright, 2003), not to mention its part in the development of the phenothiazine neuroleptic family (Kristiansen, 1989; Shen, 1999; Frankenburg and Baldessarini, 2008).

Over the years, MB has also been used in human and veterinary medicine for a number of therapeutic and diagnostic procedures including use as a stain in neuroanatomy and bacteriology, as a redox coloring agent in biochemical studies, as a targeting agent for various types of cancer such as melanoma and lung cancer, and as an antiseptic compound (for reviews, Barbosa and Peters, 1971; Wainwright and Crossley, 2002; Wainwright, 2003). One of the most common clinical applications is for treating methemoglobinemia, either inborn (Cawein et al., 1964) or induced by overexposure to drugs, to industrial chemicals such as nitrophenols, or to environmental poisons such as excessive nitrate in well water or cyanide compounds (Sills and Zinkkam, 1994). Presently, MB is used clinically in a wide range of indications, such as methemoglobinemia, ifosfamide-induced encephalopathy and thyroid surgery (Table 1). In June 2009, 14 clinical trials are registered in the United States (clinicaltrials.gov; NIH) to investigate the clinical utility of MB in areas ranging from anesthesiology to treatments of depression and psychosis (Table 2).

2. Pharmacokinetic Properties

In clinical use, MB is either dissolved in sterile water to a concentration of 10 mg/ml (1%) or administered orally in gelatin capsules to avoid staining of the oral mucous membranes and to ensure complete gastrointestinal delivery. The generally accepted therapeutic bolus dose of MB is 1–2 mg/kg body weight over 10–20 min (Harvey, 1980). In humans, mean plasma concentration of 5 μM MB was reported after intravenous bolus injection of 1.4 mg/ kg MB (Aeschlimann et al., 1996). The clinically used oral dose of MB appears to be between 50-300 mg (Herman et al., 1999). In healthy individuals, whole blood concentrations of up to 25 ng/ml were reached after oral administration of 100 mg MB (Peter et al., 2000). A recent study comparing the administration of single doses of MB (50 mg intravenously versus 500 mg orally) indicated that the absolute bioavailability of MB after oral administration was 72.3% (Walter-Sack et al., 2009). However, while oral MB results in higher intestinal and liver concentrations, intravenous administration results in higher MB concentrations in the brain (Peter et al., 2000), MB has been shown to pass the blood-brain barrier, when administered intraperitoneally (O'Leary et al., 1968), intraduodenally, and intravenously (Peter et al., 2000) to rats. MB has also been demonstrated to penetrate selectively certain neuronal cell types after systemic administration (Müller, 1998).

It is important to note that MB concentrations in whole blood have been found to be 4 to 5-fold higher than in plasma, suggesting that MB binds to and is taken up by blood cells (Peter et al., 2000; Rengelshausen et al., 2004; Buchholz et al., 2008). Thus, whole blood measurements of MB may not reflect its bio-phase concentrations. In addition, MB binds to bovine serum albumin with a stoichiometry of 1:1 and with a dissociation constant of 2.90 μM (Buchholz et al., 2008). Thus, not surprisingly, MB has an exceedingly high volume distribution of 21.0 l/kg in rabbits (Kozaki and Watanabe, 1981). However, pharmacokinetic properties of MB vary significantly depending on the dose of MB and animal species involved (DiSanto and Wagner; 1972; Kozaki and Watanabe, 1981; Burrows, 1984).

Both intravenous and oral administrations of MB demonstrate multi-compartmental pharmacokinetics with a terminal plasma half-life of 5-7 h (Peter et al., 2000). The oral absorption of MB is reported to be 53–97% with plasma peak concentrations after 30–60 min (DiSanto and Wagner; 1972). About 65-85% of MB is reduced in erythrocytes and peripheral tissues to the leucoMB form (DiSanto and Wagner; 1972). MB and leucoMB are predominantly excreted in urine, but also in bile and feces (Peter et al., 2000; Clifton and Leikin, 2003). MB causes a green-blue discoloration of the urine (DiSanto and Wagner; 1972), which can also be observed in saliva and bile, and disappears within a few days after its discontinuation.

3. Physicochemical Properties and Mechanisms of Actions

Methylene blue (tetramethylthionine chloride, $C_{16}H_{18}ClN_3S$) is a heterocyclic aromatic dye, a member of thiazine dyes. It occurs as odorless dark blue crystals and is soluble in water and chloroform but only sparingly so in alcohol. In its oxidized state, the color of MB is blue due to the fact that the phenothiazinium molecule absorbs visible light strongly in the region of 600–700 nm, thus allowing the remainder of the visible spectrum (350–600 nm) to be transmitted. In its reduced form (leucoMB), it is colorless and does not absorb light in the visible region (Lee and Mills, 2003). LeucoMB is uncharged, lipophilic, and enters cells by diffusion across the plasma membrane where it can be re-oxidized and thus sequestered within the cells (May et al., 2003). Oxidized MB and leucoMB together form a reversible oxidation-reduction system or electron donor-acceptor couple (Figure 1). The redox-indicator properties of this couple have been used extensively in the quantitative analysis of a large number of reducing agents, such as glucose and ascorbic acid (Wainwright, 2003). In clinics, the redox properties of MB have been utilized in treatments of methemoglobinemias and ifosfamide-induced encephalopathy (see section 7). MB is the first-line treatment for congenital, occupational, and drug induced methemoglobinemias (Bradberry, 2003). MB acts as an electron donor in the non-enzymatic reduction of methemoglobin. An NADPH methemoglobin reductase catalyses the reduction of MB to leucoMB. This reaction transfers electrons to methemoglobin non-enzymatically, restoring functional hemoglobin and MB (Bradberry, 2003).

MB absorbs energy directly from a light source and then transfers this energy to molecular oxygen creating singlet oxygen ($^1O_2$). Singlet oxygen is extremely electrophilic and can oxidize directly electron rich double bonds in biological molecules and macromolecules. For this reason, MB has been utilized as a photosensitizer in cancer treatment and protection of serum from viral agents (Wainwright 2000; Tardivo et al., 2005). The antifungal and antibacterial activity of MB can be increased significantly by light activation (Wainwright and Crossley 2002). In photosensitization, MB can have two different mechanisms of action. Excitation of MB by light can induce singlet and triplet stages of the molecule and transfer the energy through either electrons (Type I mechanism) or energy (Type II mechanism). The Type I mechanism can lead to the formation of hydroxyl radicals and lipid hydroperoxides. The Type II mechanism proceeds through singlet oxygen resulting mainly in breakages of nucleic acids, mostly at the guanosine site (Wainwright, 2000). Photosensitization reactions induced by MB excitation are known to cause damage to several biomolecules. Damage to nucleic acids, proteins and lipids have been described in the literature. Especially lipid peroxidation, induced by MB photosensitization, has detrimental effects on membrane integrity, leading to a loss of fluidity and alterations on the functions of several ion channels, receptors, and transporters (see section 8). This damage is thought to be triggered both by type I and type II mechanisms and has been comprehensively described in earlier reviews (Tuite and Kelly, 1993; Tardivo et al., 2005).

However, MB also exhibits antioxidant effects (McCord and Fridovich, 1970; Kelner et al., 1988; Salaris et al., 1991; Riedel et al., 2003). As mentioned earlier, MB can readily accept and donate electrons from and to a variety of compounds, allowing it to be either prooxidant or antioxidant under different conditions. For example, MB inhibits superoxide production by serving as an artificial electron acceptor by diverting electron flow away from the enzyme sites of various oxidases where molecular oxygen is converted to superoxide radicals (Salaris et al., 1991; Riedel et al., 2003). Furthermore, in HepG2 cells, MB induces phase-2 antioxidant defense enzymes such as thioredoxin reductase 1 and NADPH quinone oxidoreductase (Atamna et al., 2008).

In pharmacological studies, 1-10 μM MB is used routinely to inhibit soluble guanylate cyclase (sGC) for the analysis of cGMP-mediated processes (Moncada and Higgs, 2006). MB also directly inhibits both constitutive and inducible forms of nitric oxide synthase (NOS) by oxidation of ferrous iron bound to the enzyme (Mayer et al., 1993; Volke et al., 1999). It inactivates nitric oxide (NO) by generation of superoxide anions (Wolin et al., 1990; Marczin et al., 1992). MB is likely to be a more specific and potent inhibitor of NOS than of sGC, since direct NO-donating compounds in the presence of MB can still partially activate cGMP signaling pathways (Mayer et al., 1993).

However, in recent years, it has become clear that several pharmacological actions of MB are mediated by mechanisms other than inhibition of the cGMP pathway. Regardless of its mode of action, MB proved invaluable in various branches of medicine and biology. In the following chapters, special emphasis will be given to the cellular and molecular basis of MB actions in the nervous system.

3. Adverse Effects

In adults, large doses of MB (>7.0 mg/kg) administered intravenously have been reported to cause nausea, abdominal and chest pain, cyanosis, methemoglobinemia, sweating, dizziness, headache, and confusion (Harvey, 1980). In neonates, MB toxicities include hemolytic anemia, respiratory distress, and phototoxicity (Cragan, 1999). MB administration during breast feeding is also discouraged since MB easily crosses the blood-milk barrier (Ziv and Heavner, 1984). However, a review of the literature indicates that, especially in neonates, the most likely cause for MB toxicity is excessive dose administration (Albert et al., 2003). From the toxicological point of view, MB has an enviable safety record. In one notable pediatric case report (Blass and Fung, 1976), a child was given a dose that was 16 times the recommended maximum. The child's skin was intensely blue but there were no other documented ill effects.

Intra-amniotic MB instillation has also been reported to cause Heinz body hemolytic anemia (Sills and, Zinkham, 1994) and liver damage (McFadyen, 1992). Importantly, MB has been associated with a high percentage of fetal intestinal atresia when used in the second trimester of pregnancy (Dolk, 1991; van der Pol et al., 1992; Gluer; 1995). In preclinical studies the intra-amniotic instillation of MB produced teratogenic effects including cleft palate, digit malformations, increased incidence of fetal mortality, and neuronal tube malformations (Iyengar and Lal, 1985; Tiboni et al., 2001). Intra-amniotic MB injection is currently not recommended in obstetrics due to its association with above mentioned teratogenic effects (for a review Cragan, 1999).

In preclinical *in vitro* studies, MB is employed at concentrations ranging from 0.1 nM to 10 μM and toxic effects are reported at levels higher than 100 μM. The oral LD_{50} of MB has been estimated as 1,180 mg/kg in rats and 3,500 mg/kg in mice (Lewis, 1992); the intraperitoneal LD_{50} as 150 mg/kg in mice and 180 mg/kg in rats; and the intravenous LD_{50} as 77 mg/kg in mice, 1,250 mg/kg in rats, and 42.3 mg/kg in sheep (Burrows, 1984; Lewis,

1992; Cragan, 1999). The results of preclinical toxicity studies by the National Toxicology Program (2008) indicated that long-term MB administration (1 to 24 months) in rodents is associated with lymphomas as well as various intestinal malignancies.

5. Methylene Blue and Neurohistology

In 1886, Ehrlich carried out the first MB staining of nervous tissue by injecting it intravenously into a living animal (Ehrlich, 1886). In the following years, various methods for both vital and supra-vital MB staining of neuronal structures were developed and employed in numerous studies (for a review Barbosa and Peters, 1971). Importantly, camera lucida drawings used for Cajal's neuron theory are also based on his early MB-studies (for a review Garcia-Lopez et al., 2007). MB has been used to locate muscle spindles (Hines and Tower, 1928), cutaneous nerve endings (Weddell, 1941), motor end plates (Coers, 1952; Cheng, 1954) and nodes of Ranvier (Müller, 1992). MB has a high affinity towards nervous tissue and binds indiscriminately to motor, sensory and autonomous nerve fibers (Kristiansen, 1989). However, it selectively stains certain subpopulations of neurons in the brain, e.g. cerebellar Purkinje cells, where it binds to their dendritic spines (Müller, 1992) or polymorphic nerve cells in the hippocampal cornu ammonis, after supra-vital (intracardiac injection in deeply anesthetized animals) administration (Müller, 1998) and has been employed routinely for the staining of neuronal structures in clinical and histochemical studies (Frimer et al., 1970; Kristiansen, 1989; Müller, 1990; 1998; Seif et al., 2004). At the ultrastructural level, it was shown that MB appears as electron-dense precipitates staining the cytoplasm and chromatin homogeneously. Moreover, MB accumulates in the shape of large, drop-like structures in the cytoplasm and binds to material at the plasma membrane, possibly belonging to perineuronal nets (Müller, 1998).

Pharmacokinetic studies of its organ distribution in rodents have shown that the concentration of MB is 10–20 times higher in the brain than in the circulation one hour following systemic administration, indicating a rapid and extensive accumulation of MB in the nervous system (Kristiansen, 1989; Peter et al., 2000). The cellular mechanism(s) underlying the high affinity uptake of MB into nervous tissue is not completely elucidated. It is likely that multiple factors play a role in this process. The presence of a sulfur atom in the MB molecule has been suggested as the reason for its high affinity to neural structures (Müller, 1998). MB is a cation whereas the reduced (leuco) form has a pKa of 5.8, resulting in very low ionization (3%) at physiological pH. It has been suggested that MB is reduced to its uncharged leuco-form by a reductase associated with the plasma membrane, immediately before entering nerve cells, followed by intracellular reoxidation to the oxidized (blue) form (Müller, 1998). Ehrlich suggested that neuronal structures showed a high affinity for the reduced (*leuko*) form of MB and demonstrated this phenomenon by exposing tissue sections to air or by treating them with iron chloride. This resulted in the return of the blue stain (Ehrlich, 1886). Color alterations between oxidized and reduced forms of MB have also been utilized successfully in monitoring neuronal activity levels (Fischer and Zeman, 1959).

6. Methylene Blue in Anesthesiology

Local applications of MB at high concentrations not only stain nerve endings and fibers but also disrupt their function. This property of MB has been utilized for the treatment of sensory pathologies such as pain and itching (Kristiansen, 1989). In fact, MB was first employed as an analgesic in 1890 (Ehrlich and Leppmann, 1890). Ehrlich, together with the German psychiatrist Arthur Leppmann, used this dye successfully in the treatment of neuritic and rheumatic diseases. Local MB applications have been used for the functional blockade of sensory nerve endings in the treatment of pain (Zakaria et al., 2005; Tan and Seow-Choen, 2007; Peng et al., 2007) and intractable itching (Wolloch and Dintsman, 1979;

Eusebio et al., 1990; Mentes et al., 2004; Sutherland et al., 2009). Furthermore, the hyperalgesia induced by intrathecal injections of prostaglandin E2 and prostaglandin F2 was inhibited by low μM concentrations of MB (Minami et al., 1995; Ferreira and Lorenzetti, 1996). However, intrathecal application of MB is absolutely contraindicated; in cats, epidural injections of MB induced paraplegia, axonal swelling, and inflammation of the leptomeninges (Poppers et al., 1970). Several earlier clinical studies have described neurotoxic adverse effects of intrathecal MB; neurological sequelae included quadriplegia, paraplegia, radiculopathy, cauda equina syndrome, encephalopathy, optic neuritis, and meningeal irritation (Arieff and Pyzik, 1960; Evans and Keegan, 1960; Schultz and Schwartz, 1970; Steiner and Steiner-Milz, 1986). Although the mechanism for these detrimental effects of MB is not clear, it is likely that anatomical regions with limited blood perfusion, such as the spinal cord, are highly sensitive to the well-known vasoconstrictive effects of MB (Sharr et al., 1978). Furthermore, *in vitro* exposure of organotypic slice cultures of the immature rat cerebellum to high MB concentrations resulted in a progressive destruction of differentiating cells (Garthwaite and Garthwaite, 1988), suggesting that MB may have additional neurotoxic effects at concentrations above 100 μM.

In addition to its local analgesic actions, MB also decreases minimum alveolar concentration of general anesthetics such as sevoflurane (Masaki and Kondo, 1999), isoflurane (Vutskits et al., 2008), and the propofol (Licker et al., 2008). In fact, phenothiazine-class compounds were employed initially to potentiate the effects of anesthetics and sedatives (for reviews Kristiansen, 1989; Frankenburg and Baldessarini, 2008).

7. Methylene Blue and Ifosfamide Encephalopathy

In neuro-oncology, MB has recently been introduced into the treatment of ifosfamide-induced encephalopathy (Küpfer et al., 1994; Zulian et al., 1995; Aeschlimann et al., 1996; Pelgrims et al., 2000). Ifosfamide is an alkylating agent used on a wide range of solid tumors (sarcomas and germ cell tumors) and hematologic malignancies. However, neuropsychiatric toxicity, commonly known as ifosfamide encephalopathy, is one of the most serious adverse effects of ifosfamide, occurring in 5-30 % of patients treated with this agent (Alici-Evcimen and Breitbart, 2007). Clinical symptoms may range from mild confusion to stupor, coma, hallucinations, seizures, and death. Both oral and intravenous administrations of MB have been shown to prevent and reverse ifosfamide-induced encephalopathy (Küpfer et al., 1994; Ferrero et al., 1995). For this purpose, prophylactic use of MB has also been suggested for preventing the recurrence of encephalopathy by subsequent administrations of ifosfamide (Küpfer et al., 1994; Alici-Evcimen and Breitbart, 2007; Di Cataldo et al., 2009).

The pathophysiology of ifosfamide encephalopathy is still poorly understood. However, chloroacetaldehyde (CAA) accumulation in the nervous system and defects in mitochondrial fatty acid oxidation due to defective electron transfer to flavoproteins appear to be involved (Ajithkumar et al., 2007; Alici-Evcimen and Breitbart, 2007). Since MB has a redox potential close to that of oxygen and can be reduced by components of the electron transport chain, it is very efficient in cycling between oxidized and reduced forms of redox factors in the mitochondria (Clark et al., 1925; Ajithkumar et al., 2007). MB restores mitochondrial respiratory chain function by serving as an alternative electron acceptor, restoring hepatic gluconeogenesis by reversing NADH inhibition, preventing the transformation of chloroethylamine into CAA, and inhibiting multiple amine oxidase activities, thereby preventing the formation of CAA (Ajithkumar et al., 2007). In order to compensate for the inhibition of gluconeogenesis caused by ifosfamide, MB is usually administered in combination with a glucose infusion (Alici-Evcimen and Breitbart, 2007). In the 1930s it was suggested that defective electron transfer in cyanide poisoning can be corrected by MB

(Brooks, 1936; Wendel, 1935). These findings have led to the development of the current antidotal treatment of cyanide poisoning (Gracia and Shepherd, 2004).

8. Methylene Blue and the Neuroendocrine System

MB has been shown to modulate the physiological actions of hormones involved in the hypothalamo-pituitary-peripheral axis (Nedvídkova et al., 1995; 2000; 2001). For example, MB raises blood thyroxine and causes a feedback decrease in the serum thyroid stimulating hormone level (Nedvídkova et al., 1995). It appears that an MB-induced increase in thyroid peroxidase activity enhances the iodination of thyronines, with subsequent increases in the synthesis of thyroxine (Nedvídkova et al., 2000). As a redox modulating agent, MB acts as an alternative electron acceptor in the mitochondrial oxidation chain and in tissue oxidases, particularly xanthine oxidase. MB also inhibited the activity of aldehyde dehydrogenases in human erythrocyte and leukocytes, and the rat liver mitochondria (Helander et al., 1993). Although this effect was suggested to protect against the metabolic redox effects of ethanol (Ryle et al., 1985), it has been shown in cultured rat astrocytes that MB-induced acetaldehyde accumulation during chronic ethanol exposure potentiates the toxic effects of ethanol *in vitro* (Signorini-Allibe et. al., 2005). *In vivo* studies in rats indicate that MB significantly increases the metabolism of ethanol to CO_2, indicating that it can speed up ethanol elimination (Vonlanthen et al., 2000). However this effect was not observed in humans, when MB was given in a dose (50 mg orally) that can be safely administered (Vonlanthen et al., 2000)

Similar to thyroid hormone, the actions of estrogens are also modulated by MB (Hirsch et al., 1989). Estradiol-induced adenohypophyseal enlargement and the enhancement of prolactin secretion are antagonized by MB (Nedvídkova et al., 2000; 2001). Treatment with MB also prevents estradiol-induced decreases in anterior pituitary dopamine levels and increases in D2 receptor number. MB alone reduces anterior pituitary weight, increases anterior pituitary dopamine concentrations and decreases anterior pituitary D2 receptor number with a corresponding reduction in its affinity (Nedvídkova et al., 2000; 2001). It is likely that inhibition of estradiol actions in the anterior pituitary by MB is mediated by elevations in dopamine levels induced by the actions of MB on receptor binding and monoamine oxidase activity (see below).

9. Molecular Targets of Methylene Blue

At the cellular level, MB modulates the functions of various integral membrane proteins involved in transports of solutes such as glucose (Louters et al., 2006; Scott et al., 2009) and ions such as Na^+, K^+, and H^+ (Puppi et al., 1986; Sanders et al., 1989; Visarius et al., 1997; Shah et al., 2006; Furian et al., 2007). In addition, the functions of voltage-sensitive Na^+ (Oxford, 1977; Starkus et al., 1984; 1993; Kress et al., 1997), Ca^{2+} (Thuneberg, 1990) and Ca^{2+}-activated K^+ channels (Nemeth et al., 1985; Stockand and Samson, 1996; Saitow and Nakaoka, 1997) are also modulated by MB. As a result of MB actions on ion channels, excitability of neurons is altered significantly. Depolarizing effects of MB on neuronal structures have been demonstrated in several earlier studies (Burmistrov et al., 1967; Nozdrachev et al., 1984; Nemeth et al., 1985; Kress et al., 1997). Inhibition of Ca^{2+}-activated K^+ channels (Nemeth et al., 1985), activation of Ca^{2+} channels (Saitow and Nakaoka, 1997), and facilitation of Na^+ channel inactivation (Oxford, 1977; Kress et al., 1997) are suggested to be possible mechanisms for MB-induced depolarizations. It appears that the actions of MB, reported in the majority of these studies, are not mediated by the cGMP pathway. Molecular mechanisms mediating the effects of MB on these ion channels are complex and depend on multiple factors including the route of MB application (Oxford, 1977; Armstrong and Croop, 1982; Starkus et al., 1984; 1993), light exposure (Pooler, 1968;

Starkus et al., 1993; Kress et al., 1997), membrane potential (Valenzeno et al., 1993) and the redox state of cells (Nanasi and Dely, 1995). Among these factors, light exposure deserves mention since it is particularly important during *in vitro* experiments in which various light sources are used routinely. Some of the depolarizing actions of MB can be induced by visible light illumination. When exposed to light, MB becomes photosensitized, leading to the release of cytotoxic, highly active, and short-lived oxygen-derived species such as singlet oxygen (see section 3). These photooxidation products are known to alter the functional properties of several enzymes, proteins, and ion channels by oxidation of amino acids such as histidine, methionine, tryptophan, and tyrosine (Weil et al., 1951; Chatterjee and Noltmann, 1967). Furthermore, these cytotoxic species induce direct oxidative damage to cellular organelles and promote apoptosis (for a review, Triesscheijn et al., 2006).

10. Methylene Blue and Neurotransmitter Systems

MB influences neuronal communication by altering cholinergic (Cook, 1926; Pfaffendorf et al., 1997), monoaminergic (Ramsay et al., 2007), and glutamatergic (Vutskits et al., 2008) synaptic neurotransmission both in the central and the peripheral nervous systems. MB modulates the cholinergic system at multiple levels. At the receptor level, MB competitively displaces the binding of muscarinic acetylcholine receptor antagonists such as [^{3}H]quinuclidinyl benzylate in cardiac myocytes (K_D= 187 nM; Abi-Gerges et al., 1997) and [^{3}H]-N-Methylscopolamine (K_D= 550 nM; Pfaffendorf et al., 1997) in cardiac membrane homogenates. However, cognitive deficits induced by scopolamine (a muscarinic antagonist) are reversed by MB (Deiana et al., 2009), suggesting that MB can still exert beneficial effects when muscarinic receptors are blocked.

At the synaptic level, MB increases acetylcholine concentrations by inhibiting the function of acetylcholinesterase (AChE), an enzyme that hydrolyzes acetylcholine. In fact, MB shares this action mechanism with other AChE inhibitors presently used as long-term symptomatic treatment in patients with Alzheimer's Disease (see below). Earlier studies indicated that AChE activity in cow erythrocytes was competitively inhibited by MB with an IC$_{50}$ of 0.57 μM (Augustinsson, 1950). Interestingly, the extent of inhibition by MB was decreased significantly when the incubation time of MB was increased. Since the reduced form of MB was ineffective on AChE activity, it was concluded that the formation of leucoMB during prolonged incubation periods causes decreased efficacy of MB in *in vitro* assay systems. In a more recent study, MB inhibited the esterase activity of human plasma and bovine AChE concentration-dependently with IC$_{50}$ values of 1.1 μM, and 0.42 μM, respectively (Pfaffendorf et. al., 1997).

The second major form of cholinesterase is butyrylcholinesterase (BuChE, also known as pseudocholinesterase; plasma cholinesterase, or formally, acylcholine acylhydrolase), found primarily in the liver and to a lesser extent in the brain. The two forms, AChE and BuChE, differ genetically, structurally, and in their kinetics. Butyrylcholine is not a physiological substrate in the mammalian brain, which makes the function of BuChE difficult to interpret. Nevertheless, MB inhibits the activity of BuChE in a concentration-dependent manner with IC$_{50}$ values ranging between 0.4 μM and 5.3 μM (Pfaffendorf et. al., 1997; Kucukkilinc and Ozer, 2007, Yucel et al., 2008). Finally, in addition to MB, several other phenothiazine derivatives also block the activity of BuChE with K_d values ranging from 4 nM to 10 μM (Radic et al., 1993; Debord et al., 2002; Darvesh et al., 2005; 2007). It appears that the extent of MB inhibition of cholinesterase activity differs significantly in an isoenyme- (AChE versus BuChE) and species-dependent manner (Augustinsson, 1950; Pfaffendorf et. al., 1997).

MB can also affect the metabolism of choline, a precursor for acetylcholine synthesis, by inhibiting the activity of choline oxidase (COx), an enzyme that oxidizes choline to betaine aldehyde (Tacal and Ozer, 2006). COx activity has been detected in the brain (Li et al., 2007) and intraperitoneal injection of this enzyme impairs learning and memory in mice (Ikarashi et al., 2000). However, the physiological importance of MB inhibition of COx is not clear since choline deficiencies are completely compensated in a mouse knock-out model (Li et al., 2007).

MB also modulates the glutamatergic system. In rat hippocampal slices, glutamatemediated synaptic transmission is abolished by relatively high concentrations (5-50 μM) of MB (Vutskits et al., 2008). Although glutamatergic transmission plays important roles in learning and memory and MB is known to improve cognitive functions in animal models (Martinez et al., 1978; Callaway et al., 2002, 2004; Gonzalez-Lima and Bruchey, 2004; Wrubel et al., 2007), currently there are no studies on the actions of MB on glutamate receptors, neither ionotropic nor metabotropic.

Both glutamate and dopamine have been implicated in the pathogenesis of psychoses and MB has been the lead compound for the development of classical antipsychotics such as chlorpromazine (for reviews; Kristiansen, 1989; Wainwright, 2003). Considering the similarities in the chemical structures of MB and antipsychotics of the phenothiazine class, it is likely that MB modulates the activity of dopamine receptors. Although there are no current studies on the actions of MB on dopamine receptors, the antipsychotic effects of MB have been known for more than a century (Bodoni, 1899). Knowing that MB acts on nerve cells, Bodoni used MB to effectively calm psychotic agitation, also reporting that many other physicians in the Genova region were administering it for the same indication (Healey, 2002). More recent preclinical (Klamer et al., 2004) and clinical studies have confirmed the beneficial effects of MB in the treatment of psychotic disorders (Narsapur and Naylor, 1983; Thomas and Callender, 1985; Naylor et al., 1986; 1988; Deutsch et al., 1997).

MB has also beneficial effects in brain disorders traditionally linked to disturbances in the serotonergic system. For example, MB showed antidepressant actions in clinical trials (Naylor et al., 1986; 1987). More recently, anxiolytic and antidepressant-like activities of MB were reported in various behavioral animal studies (Eroglu and Caglayan, 1997; De-Oliveira and Guimaraes, 1999; Volke et al., 2003; Patil et al., 2005). Cellular mechanisms mediating antidepressant actions of MB have been investigated in several earlier studies. Wegener et al. (2000) demonstrated that NOS inhibitors including MB increased extracellular levels of serotonin (5-HT) and dopamine in the rat ventral hippocampus after local or systemic administration, whereas the NO precursor *L*-arginine had the opposite effect. In another study, 7-nitroindazole, a neuronal NOS inhibitor, potentiated the effect of bupropion, a dopamine reuptake inhibitor, and this effect was antagonized by *L*-arginine (Dhir and Kulkarni, 2007). Thus, these studies suggest that endogenous NO exerts a negative control over monoamine levels and that inhibition of NO synthesis may underlie the antidepressant action of MB.

However, contradictory evidence also exists concerning the influence of NO on monoamine levels. For example, *L*-arginine has been shown to induce dopamine release from the striatum and to increase the extracellular levels of 5-HT and dopamine in the medial preoptic area *in vivo* (Strasser et al., 1994; Lorrain and Hull, 1993). Therefore, in addition to the cGMP pathway, additional molecular targets are likely to play a role in the antidepressant actions of MB.

The inhibition of monoamine oxidase (MAO) activity by MB has been known for many decades (Philpot, 1937; Philpot and Cantoni, 1941; Imaizumi et al., 1959; Ehringer et al.,

1961; Cotzias et al., 1974). It was reported that MB (0.5 μM) potently inhibited the activity of MAO in guinea pig liver homogenates and potentiated the vasoconstriction induced by tyramine (Imaizumi et al., 1959). Binding of MB to MAO in mouse brain has also been shown in histochemical studies (Cotzias et al., 1974).

5-HT is considered to be a relatively specific substrate for MAO-A, but not for MAO-B and the pharmacological profiles of antidepressants are closely associated with selective inhibition of MAO-A. Recent studies indicated that MB selectively inhibits the function of MAO type A, with IC_{50} values ranging from to 27 to 180 nM (Oxenkrug et al., 2007; Ramsay et al., 2007). Induction of melatonin biosynthesis by MB and antidepressant effects related to MB inhibition of MAO-A have also been shown (Oxenkrug et al., 2007).

11. Methylene Blue and Serotonin Toxicity

Recently, the effects of MB on monoaminergic transmission have attracted considerable attention in unfolding the mechanisms of prolonged CNS side effects of MB observed in some patients after surgical procedures. For example, preoperative intravenous administration of MB is a widely used practice to identify neuronal structures during parathyroid surgery (Dudley et al., 1971; Gordon et al., 1975; Sherlock and Holl-Allen, 1984; Muslumanoglu et al., 1995; Kuriloff and Sanborn, 2004; Sweet and Standiford, 2007). However, during the past few years, an emerging number of clinical observations have suggested that MB infusion during parathyroid surgery can induce prolonged disorientation, dizziness, headache, tremors, seizures, and mental confusion during the postoperative period (Martindale and Stedeford, 2003; Bach et al., 2004; Kartha et al., 2006; Mathew et al., 2006; Majithia and Stearns, 2006; Khan et al., 2007; Ng et al., 2008). It was found that these symptoms usually occur in patients who are also under medication of serotonin reuptake inhibitors (SRIs). Since MB is a relatively specific inhibitor of MAO-A (Aeschlimann et al., 1996; Oxenkrug et al., 2007; Ramsay et al., 2007) in patients administered SRIs such as citalopram, clomipramine, duloxetine, imipramine, sibutramine, and venlafaxine, intravenous infusion of MB, especially at doses exceeding 5 mg/kg (Gillman, 2006; 2008; Ramsay et al., 2007; Khavandi et al., 2008), may precipitate 5-HT toxicity or 5-HT syndrome (Boyer and Shannon, 2005). For this reason, the use of MB is contraindicated in patients treated with SRIs (Parlow et al., 2008; Stanford et al., 2009).

12. Methylene Blue and Neurodegenerative Diseases

Recent clinical studies indicate that MB may also have a beneficial effect on the cognitive performance of patients with Alzheimer's disease (AD) (Wischik et al., 2008; see also Gura et al., 2008; Sullivan, 2008; Oz et al. 2009). AD, a progressive neurodegenerative disorder characterized by irreversible cognitive impairment, is the leading cause of dementia in the elderly population. Its neuropathology is characterized by senile plaques (SP), neurofibrillary tangles, and neuronal cell death (see Goedert and Spillantini, 2006; Zhu et al., 2007 for review). SP are accumulations of amyloid beta protein, which is produced by cleavage of amyloid precursor protein. Neurofibrillary tangles are intracellular cytoplasmic aggregates of hyperphosphorylated microtubule-associated tau-protein (Binder et al., 2005; Neve and McPhie, 2006). Cell death preferentially affects neurons of the basal forebrain, the main source of cholinergic innervation in the forebrain, leading to a marked reduction in cholinergic markers in the cortex. It is currently not yet clear whether improper amyloid precursor protein processing (amyloid hypothesis), abnormal tau phosphorylation (tau hypothesis) or reduced acetylcholine synthesis (cholinergic hypothesis) lies at the root of AD.

MB influences all three key neuropathological features of AD (Figure 2). Amyloid plaques are extracellular fibrillar deposits formed by aggregation of Aβ, which first forms oligomers

and then fibrils. Amyloid precursor protein is an integral membrane protein, which is metabolized by several metabolic pathways. When amyloid precursor protein is cleaved extracellularly by β or γ secretase, the amyloidogenic peptide is released, whereas α secretase generates the secreted form of amyloid precursor protein α, which prevents formation of amyloidogenic fragments (Govoni et al., 2001). MB has been reported to inhibit the formation of Aβ oligomers (Necula et al., 2007a). However, data regarding fibril formation are conflicting; both inhibition (Taniguchi et al., 2005) and promotion (Necula et al., 2007a,b) of fibril formation have been described. Recently, quinacrine, a closely related derivative of MB and other thiazine-related dyes, was also reported to inhibit Aβ fibril formation (Necula et al., 2007b;Dolphin et al., 2008).

Acetylcholine plays an important role in cognitive functions, especially memory, and depletion of cholinergic markers is an invariable feature of AD. Because AChE inhibitors increase synaptic acetylcholine levels, thereby enhancing cholinergic neurotransmission indirectly, they are currently used in the symptomatic treatment of AD (Nordberg, 2006; Holzgrabe et al., 2007). As mentioned earlier, MB increases cholinergic transmission by inhibiting both AChE (Augustinsson, 1950) and BuChE (Kucukkilinc and Ozer, 2007; Yucel et al., 2008). BuChE has been detected in neuritic plaques and tangles occurring in the brains of AD patients. Whereas brain AChE activity decreases in the course of AD, BuChE activity rises progressively (Giacobini, 2003) and BuChE possibly compensates for diminished AChE activity. When BuChE is selectively inhibited in rodents, learning performance improves and β-amyloid protein levels decrease (Greig et al., 2005). It has therefore been proposed that substances which inhibit the activities of both AChE and BuChE are most efficient in treating advanced AD (Giacobini, 2003; Geula and Darvesh, 2004).

AChE also interacts with senile plaques by forming stable and highly toxic AChE-amyloid-Aβ complexes (Nordberg, 2006). When AChE activity is inhibited, AChE-Aβ complex formation is impaired and expression of nicotinic receptors is increased, resulting in improved cognitive performance in AD patients (for reviews Nordberg, 2006, Holzgrabe et al., 2007; Inestrosa et al., 2008).

Dyes closely related to MB have been known to stain tau and beta amyloid-related cellular pathologies in AD (Mena et al., 1995) and have recently been proposed as a neuropathological tool for the rapid diagnosis of AD in tissue imprints (Luna-Munoz et al., 2008). The idea that MB may be a potential therapeutic against AD arose from the observation that tau filaments could be dissolved by a number of phenothiazines including MB and its desmethylated derivatives Azure A, Azure B, and tolonium chloride (Wischik et al., 1996). Similarly, heparin-induced assembly of tau protein into filaments is inhibited *in vitro* by MB and several other phenothiazines such as azure A, azure B, and quinacrine mustard (Taniguchi et al., 2005). The first and fourth repeats of the microtubule-binding domain (MBD) of the tau protein are essential for this inhibitory activity of MB upon filament formation (Hattori et al., 2008).

In addition to cholinergic disturbances, alterations in serotonergic transmission have also been reported in AD (Lorke et al., 2006). Association of AD with a loss of serotonergic neurons in the raphe nuclei, a reduction in serotonergic projection fibers in the cerebral cortex, a marked depletion in cortical 5-HT, and a significant decrease in cortical neurons expressing 5-HT receptors has been shown (Lorke et al., 2006). Elevated brain 5-HT levels as a result of MB-induced inhibition of MAO-A activity (Oxenkrug et al., 2007; Ramsay et al., 2007) may also have a beneficial effect on AD.

Other biological effects of MB that are not related to neurotransmitter systems may also influence AD-related events such as the progression of this disease (Atamna et al., 2008), memory processing, and task learning (Callaway et al., 2004; Gonzalez-Lima and Bruchey, 2004). MB has been shown to accumulate within mitochondria (Hassan and Fridovich, 1979), improve mitochondrial respiration by shuttling electrons to oxygen in the electron transport chain (Lindahl and O'berg, 1961; Visarius et al., 1997), and inhibit the production of superoxide (Kelner et al., 1988; Salaris et al. 1991). Recently, MB has been suggested to have antisenescence properties by interacting with specific mitochondrial electron carriers in its oxidized and reduced forms (Atamna et al., 2008).

Effects of MB on mitochondrial functions have also been utilized in ischemic and hypoxic conditions. MB shows significant neuroprotective effects in ischemia-reperfusion models of the brain (Wiklund et al., 2007) and the spinal cord (Bardakci et al., 2006). Similarly, retinal neurodegeneration induced by rotenone, an inhibitor of mitochondrial enzyme complex I (Zhang et al., 2006), and neurotoxic effects of methylmalonate, an inhibitor of mitochondrial enzyme complex II (Furian et al., 2007), are significantly attenuated by MB.

Transmissible spongiform encephalopathies (TSE) are fatal and incurable neurodegenerative disorders caused by prions, a class of proteins that target predominantly the CNS. Several phenothiazines closely related to MB (Amaral and Kristiansen, 2001) have been investigated for the treatment of TSEs, which include Creutzfeldt-Jakob disease in humans, bovine spongiform encephalopathy in cattle, and scrapie in sheep. Although MB was found to be ineffective in *in vitro* studies (Korth et al., 2001), quinacrine, a derivative of MB (Korth et al., 2001; Spilman et al., 2008), and chlorpromazine, closely related to MB (Dees et al., 1985; Korth et al., 2001; Kocisko et al., 2003), inhibited the conversion of soluble prion protein into the protease-resistant form and prion protein-related cellular toxicities. However, *in-vitro* effects of quinacrine vary significantly depending on the tissues, animal species, and experimental conditions (Cronier et al., 2007) and the efficacy of quinacrine has been questioned in a recent clinical study (Collinge et al., 2009).

13. Conclusions

In summary, MB has a broad range of targets encompassing multiple neurotransmitter systems, ion channels, and enzymes involved in various physiological functions of the nervous system. It appears that many of the biological effects of MB are closely associated with its unique physicochemical properties, including its redox characteristics, ionic charges, and light spectrum characteristics. MB has a high solubility in aqueous media; preclinical and clinical studies demonstrate a low toxicity profile (Küpfer et al., 1994; Riha et al., 2005). In addition, its ability to permeate cellular membranes and to cross the blood-brain barrier (Peter et al., 2000) makes MB attractive as a potential therapeutic agent. Recent clinical investigations on the biological effects of MB range from new trials on malaria treatment (Coulibaly et al., 2009) to the therapy of depression and Alzheimer's disease. However, there are still many open questions regarding the effect of MB in CNS disorders and further studies elucidating the molecular and cellular targets of MB actions are needed.

Acknowledgments

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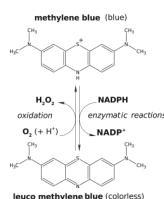
Figure 1.
Methylene blue (MB) as a redox-cycling substrate for various reducing and oxidizing substances. The reducing agents such as NADPH convert MB to leucoMB, which is then oxidized by O_2. Each reaction cycle, catalyzed by the MB-enzyme ensemble, leads to the consumption of NADPH and O_2 and to the production of reactive oxygen species, predominantly to H_2O_2 (modified from Buchholz et al., 2008).
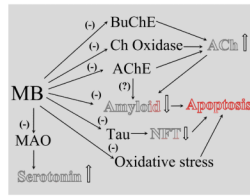
Figure 2.
A schematic view of methylene blue (MB) actions with potential beneficial effects on the progression of Alzheimer Disease. Pathological hallmarks and key neurotransmitter changes of Alzheimer Disease are highlighted. Abbreviations: AChE, acetylcholine esterase; BuChE, butyrylcholine esterase; Choline Ox, Choline oxidase; MAO, monoamine oxidase; NFT, neurofibrillary tangles.

Table I

Clinical indications of methylene blue	Dose	References
Methemoglobinemias	1-2 mg/kg I.V.	Bradberry, 2003
Ifosfamide-induced encephalopathy	50 mg IV every four hour till symptoms resolve	Küpfer et al., 1994; Alici-Evcimen and Breitbart, 2007
Treatment of vasoplegic syndrome	2 mg/kg, 20-min infusion time	Shanmugam, 2005
Parathyroid imaging	3-7.5 mg/kg I.V.	Dudley et al., 1971; Gordon et al., 1975; Rowley et al., 2009
Sentinel lymph node biopsy	Local application of 1-5 ml of 1% MB	Varghese et al., 2007
Treatment of malaria	10 mg/kg twice a day orally	Coulibaly et al., 2009

Table II

Potential applications of methylene blue in clinical neuroscience	Dose	References
Alzheimer Disease	10 nM-12 μM *in vitro*	Wischik et al., 1996; Taniguchi et al., 2005; Necula et al., 2007a,b; Atamna et al., 2008; Hattori et al., 2008
Depression and anxiety	15 mg/day oral in patients 2-60 mg/kg I.P. in rats	Naylor et al., 1986; 1987; Eroglu and Caglayan, 1997; De-Oliveira and Guimaraes, 1999; Volke et al., 2003; Patil et al., 2005
Psychosis	32-100 mg/kg I.P. in rats; 100 mg oral twice daily or 520 mg oral daily in patients	Narsapur and Naylor, 1983; Thomas and Callender, 1985; Naylor et al., 1986; 1988; Deutsch et al., 1997
Cognitive deficits	0.15-4 mg/kg I.P. in rats	Martinez et al., 1978; Callaway et al., 2002, 2004; Gonzalez-Lima and Bruchey, 2004; Wrubel et al., 2007; Deiana et al., 2009
Neuroprotection	70 μg/kg local injection in rats	Zhang et al., 2006; Rojas et al., 2009
Pain	1 ml of 1% MB locally in humans 20 mg/kg I.P. in rats	Zakaria et al., 2005; Tan and Seow-Choen, 2007; Peng et al., 2007
Intractable itching	10-15 ml of 0.5-1% MB locally in patients	Wolloch and Dintsman, 1979; Eusebio et al., 1990; Mentes et al., 2004; Sutherland et al., 2009