Review

"Lest we forget you — methylene blue . . ."

R. Heiner Schirmer[a], Heike Adler[a], Marcus Pickhardt[b,c], Eckhard Mandelkow[b,c,*]

[a] *Center of Biochemistry (BZH), University of Heidelberg, Heidelberg, Germany*
[b] *Max-Planck-Unit for Structural Molecular Biology, Hamburg, Germany*
[c] *DZNE, German Center for Neurodegenerative Diseases, Bonn, Germany*

Abstract

Methylene blue (MB), the first synthetic drug, has a 120-year-long history of diverse applications, both in medical treatments and as a staining reagent. In recent years there was a surge of interest in MB as an antimalarial agent and as a potential treatment of neurodegenerative disorders such as Alzheimer's disease (AD), possibly through its inhibition of the aggregation of tau protein. Here we review the history and medical applications of MB, with emphasis on recent developments.
© 2011 Elsevier Inc. All rights reserved.

Keywords: Methylene blue; History; Medical application; Alzheimer's disease; tau aggregation inhibitor; malaria therapy; prodrug of azure B

1. Introduction

The 2008 International Congress on Alzheimer's Disease (ICAD) in Chicago disappointed many hopes for treatments of Alzheimer's disease that aimed at reducing the amyloid burden in the brains of patients (discussed previously on the AlzForum, see www.alzforum.org, 30 July 2009). At the same time, new expectations were raised by reports on treatments designed to reduce the neurofibrillary pathology of tau protein. One prominent advocate of this approach was Claude Wischik who presented data arguing that the compound methylene blue (MB) could reduce the aggregation of tau and thereby slow down the disease (Hattori et al., 2008; Wischik et al., 1996, 2008). This prompted an intense public debate on the pros and cons of the "blue wonder" (Gura, 2008). However, it was often forgotten that methylene blue, the

first synthetic drug, had already a 120-year history in several areas of medicine.

2. Biochemistry of MB

MB is a tricyclic phenothiazine drug (Wainwright and Amaral, 2005). Under physiological conditions it is a blue cation which undergoes a catalytic redox cycle: MB is reduced by nicotinamide adenine dinucleotide phosphate (NADPH) or thioredoxin to give leucoMB, an uncharged colorless compound. LeucoMB is then spontaneously reoxidized by O_2 (Fig. 1). The typical redox-cycling of MB in vivo can be illustrated in vitro using the famous blue bottle experiment: MB is visibly reduced by glucose to give leucoMB and then, by opening the lid of the bottle it is reoxidized by atmospheric O_2: the color comes back. After closing the lid, there is a lag phase and then MB is reduced again. Analogous phenomena have been observed by pathologists at autopsies (Tan and Rodriguez, 2008; Warth et al., 2009). MB is excreted in the urine as a mixture of MB, leucoMB and demethylated metabolites, e.g., azure B and azure A (Gaudette and Lodge, 2005). MB-containing urine is very clear and has,

* Corresponding author at: Max-Planck-Unit, Structural Molecular Biology, c/o DESY, Notkestrasse 85, D-22607 Hamburg, Germany. Tel.: +49 40 8998 2810; fax: +49 40 8971 6822.

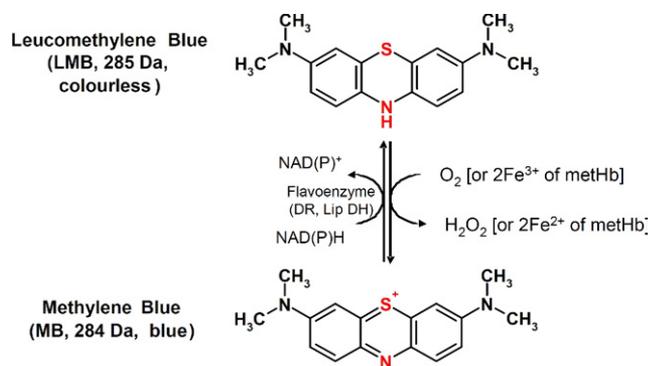*E-mail address:* mand@mpasmb.desy.de (E. Mandelkow).

Fig. 1. Methylene blue (MB) as a redox-cycling phenothiazine drug in vivo. MB is spontaneously or enzymatically reduced by NADPH and the resulting uncolored leucoMB is reoxidized by molecular oxygen (O_2) or by iron(III)-containing compounds like methemoglobin. Precious NADPH and O_2 are wasted, and hydrogen peroxide is produced in each round of the cycle. There are also pharmacologic activities of MB which do not depend on its redox properties. Azure B is monodemethylated MB, that is one of the 4 CH_3 groups shown in the formula is replaced by a hydrogen atom. Azure A is the didemethylated form at the same position, converting the $N(CH_3)_2$ to an NH_2-group.

of course, a green or blue color which disappears a few days after the last administration of MB (Guttmann and Ehrlich, 1891).

3. History of MB

MB was the very first fully synthetic drug used in medicine. In 1891 it was applied by Paul Guttmann and Paul Ehrlich for the treatment of malaria, and this application has recently been revived (Coulibaly et al., 2009; Färber et al., 1998; Vennerstrom et al., 1995). The famous Giemsa solution for staining and characterizing malaria parasites and blood cells contains MB, eosin A, and azure B as active principles (Barcia, 2007; Fleischer, 2004). Numerous other microscopic discoveries including the identification of *Mycobacterium tuberculosis* by Robert Koch and the structural organization of nerve tissues (Cajal, 1896; Ehrlich, 1886; Garcia-Lopez et al., 2007) were based on the biochemical properties of MB. Staining with MB was also the beginning of modern drug research (Kristiansen, 1989): Paul Ehrlich argued that if pathogens like bacteria and parasites are preferentially stained by MB, then this staining might indicate a specific harmful effect on the pathogen which could be exploited for fighting disease. This explains why the terms "drug" and "dye" were used synonymously until World War I.

Malaria and methylene blue played a major role also in World War II. In 1943, General Douglas MacArthur, commander of the Allied Forces in the Southwest Pacific theater stated: "This will be a long war, if for every division I have facing the enemy, I must count on a second division in the hospital with malaria, and a third division convalescing from this debilitating disease." Because of the blue urine

("Even at the loo we see, we pee, navy blue"), MB was not well liked among the soldiers (MacArthur, 1964; W. Peters, personal communication).

Going back to the beginning of the twentieth century, MB was used for a wide variety of medical and hygienic indications (Clark et al., 1925). Among others, it was added to the medication of psychiatric patients in order to study their compliance which could be monitored by the observable color of the urine. These studies led to the discovery that MB has antidepressant and further positive psychotropic effects (Bodoni, 1899; Ehrlich and Leppmann, 1890; Harvey et al., 2010). Thus MB became the lead compound for other drugs including chlorpromazine and the tricyclic antidepressants. In 1925 W. Mansfield Clark, famous for the introduction of the pH electrode and the oxygen electrode, was a coauthor of an impressive 80-page review on the application of MB in engineering, industrial chemistry, biology, and medicine. A remarkable aspect of this article is the reference list of illustrious scientists including several Nobel Prize winners — Santiago Ramon y Cajal, Robert Koch, Paul Ehrlich, Alphonse Laveran, Otto Meyerhof, and Heinrich Wieland — who contributed major papers on MB. Thus MB is an example for the high value of observations and articles that were published 100 years ago and are still relevant today.

4. Current medical indications

By 2010, there are more than 11,000 entries for "methylene blue" in the biomedical library PubMed, not counting the studies which had been published in the era not covered by PubMed. Current indications for MB that are approved by the US Food and Drug Administration (FDA) are enzymopenic hereditary methemoglobinemia and acute acquired methemoglobinemia, prevention of urinary tract infections in elderly patients (Table 1), and intraoperative visualization of nerves, nerve tissues, and endocrine glands as well as of pathologic fistulae (O'Leary et al., 1968).

Of great practical importance is also the administration of MB for the prevention and treatment of ifosfamid-induced neurotoxicity in cancer patients (Kupfer et al., 1994). Recommended doses are 3 to 6 times 50 mg/day intravenously (i.v.) or orally (p.o.) as a treatment and 3 to 4 times 50 mg/day p.o. given for prophylaxis, starting 1 day before ifosfamid-infusion and continuing still after oxazaphosphorine-treatment is finished (Pelgrims et al., 2000). Concerning inborn enzymopenic methemoglobinemia (Table 1), the treatment of the Blue People of Troublesome Creek in Kentucky — and other persons worldwide — with the *blue* drug MB was a visible success of knowledge-based medicine (Cawein et al., 1964). The rationale is that MB can be reduced to colorless leucoMB by red blood cell enzymes and that leucoMB reduces the inactive methemoglobin to give hemoglobin (Fig. 1). This conversion turns the bluish tinge of the skin to a rosy complexion — in the early 1960s

Table 1
Dosage of MB in different clinical conditions

Therapeutic indication	Dosage of methylene blue
Inherited methemoglobinemia	1×50–250 mg/day (for a lifetime) (Cawein et al., 1964)
Acute methemoglobinemia	1–2×1.3 mg/kg (i.v. over 20 minutes)
Ifosfamid-induced neurotoxicity	4×50 mg/day p.o. or i.v. (Pelgrims et al., 2000)
Prevention of urinary tract infections in elderly patients	Orally 3×65 mg/day
Vasoplegic adrenaline-resistant shock	200 mg i.v. over 1 hour followed by infusion (0.25–2 mg/kg/hour) (Warth et al., 2009)
Alzheimer's disease	3×60 mg/day (Rember™ according to Wischik et al., 2008)
Pediatric malaria	2×12 mg/kg p.o. for 3 days

Key: i.v., intravenous; MB, methylene blue; p.o., oral.

the right issue for emerging color television. Furthermore, topical MB is the treatment of choice for priapism (Van der Horst et al., 2003), and for intractable pruritus ani (Mentes et al., 2004; Sutherland et al., 2009; Wolloch and Dintsman, 1979). Recently, MB was introduced against acute catecholamine-refractory vasoplegia and other forms of shock (Shanmugam, 2005). The current interest in MB as an antimalarial compound and a potential drug in Alzheimer's disease is described below.

As to the pharmacokinetics of MB, a typical daily dosage is 200 mg MB given orally (Table 1). The apparent half-life of MB in the human body is approximately 10 hours; the bioavailability is ~73%. After the oral intake of 500 mg MB the concentration in blood peaks at 19 μM; after i.v. administration of 50 mg MB the corresponding value is approximately 2.2 μM (Walter-Sack et al., 2009); the diverging data of Peter et al. (2000) and of other authors are discussed in the same report.

The distribution among organs depends on the form of administration. When MB is given to rats intravenously, it will accumulate in the brain. MB can permeate the blood-brain barrier in rats irrespective of the administration route — intraperitoneally (i.p.) (O'Leary et al., 1968), intraduodenally, or i.v. (Peter et al., 2000; Walter-Sack et al., 2009). With patients it should never be given intrathecally. Concerning MB toxicology, a dose of 7 mg/kg given i.v. leads to severe gastro-intestinal symptoms in adult persons. For sheep, the LD50 was found to be 42 mg/kg body weight (Burrows, 1984).

There are several contraindications for MB administration. This applies, for instance, to patients taking serotonin reuptake inhibitors (Khavandi et al., 2008; Ramsay et al., 2007) and possibly to persons with certain types of hereditary glucose-6-phosphate dehydrogenase deficiency (G6PD deficiency). This enzyme provides antioxidant-reducing equivalents in the form of NADPH. G6PD deficiency in its different manifestations affects more than 500 million persons in the world and is thus the most common potentially hazardous hereditary condition.

On the other hand, positive side effects of MB acting as a tonic have also been observed; these effects are possibly due to an enhancement of mitochondrial activity (Cardamatis, 1900; Harvey et al., 2010; Riha et al., 2005).

5. Is MB the prodrug of azure B (monodemethyl MB)?

MB is metabolized yielding N-demethylated molecules like azure B and azure A (see Fig. 1). These compounds have pharmacological effects as well (Culo et al., 1991; Warth et al., 2009). For azure B and possibly other demethylated metabolites of MB as the active agents, MB is a prodrug. For biological efficiency it is probably of advantage that, in contrast to oxidized MB, oxidized azure B can assume a neutral quinoneimine form that readily diffuses through membranes. As summarized in Table 2, azure B may be responsible for pharmacological effects ascribed bona fide to MB. In the past, the distinction between MB effects and azure B effects was not made because it was not realized that there are many in vitro and in vivo conditions leading to the conversion of MB to azure B. The examples of Table 2 suggest that in most therapy-relevant parameters, azure B is superior to methylene blue; it is for instance a better inhibitor of human glutathione reductase and related enzymes. One not very conspicuous exception to this rule is the effect on growth and transmission of the malarial parasite *P. falciparum* (Table 2).

The results of Culo et al. (1991) are most impressive. Among other things, they found that, in contrast to MB, azure B was capable of protecting 10 out of 10 mice from experimentally-induced endotoxic shock and that, in another series of experiments, azure B was capable of decreasing the blood serum level of tumor necrosis factor-alpha (TNF alpha, cachexin) by a factor of 10. It is unfortunate that the dosages of MB and azure B in the latter test series were different. In comparative studies it should be remembered that the dose-response curves for both MB and azure B can have unusual shapes, indicating hormetic effects which means that at high concentrations a drug can have much less activity than at intermediate levels (Bruchey and Gonzales-Lima, 2008).

MB has been reported to be a selective inhibitor of nitric oxide (NO) synthase and of soluble guanylate cyclase, 2 enzymes involved in nitric oxide-mediated vasodilation; for this reason MB is used in catecholamine-refractory septic shock. Not yet studied are the effects of azure B on these enzymes in the NO-induced signaling pathway (Warth et al., 2009). The issue of MB versus azure B must also be

Table 2
Comparison of methylene blue with its monodemethylated metabolite azure B

Compared property or effect	MB	Azure B	References
Intracellular reduced form	Color-free leucoMB	Leuco AB	Wainwright and Amaral (2005)
Extracellular oxidized form	Dark blue cation	Dark blue cation	
Oxidized and deprotonated form	Not possible	Neutral quinoneimine	
Catalytic efficiency as substrates of glutathione reductase	$4800\ M^{-1}\ s^{-1}$	$9200\ M^{-1}\ s^{-1}$	Buchholz et al. (2008)
Inhibition of glutathione reductase	$K_i = 16\ \mu M$	$K_i = 5\ \mu M$	Buchholz et al. (2008)
Crystal structure of the ligand-glutathione reductase complex	Known at low resolution	Solved at 2 Å resolution	Schirmer et al. (2008); Fritz-Wolf (2010, personal communication)
Proportion in rat urine after giving MB	50%	50%	Gaudette and Lodge (2005)
Proportion in human liver, kidney, heart and lung at autopsy after previous MB administration	5% to 14%	86% to 95%	Warth et al. (2009)
Protection of mice from lethal LPS/endotoxic shock	Two out of 10 animals	Ten out of 10 animals	Culo et al. (1991)
Suppression of tumor necrosis factor-alpha level (TNF-alpha, cachexin)	To 10% of control	To 50% of control	Culo et al. (1991)
Growth inhibition of transplanted tumors in mice	No	Yes	Culo et al. (1991)
Inhibition of malarial parasite propagation in culture	IC50 = 4 nM	IC50 = 8 to 13 nM	Vennerstrom et al. (1995)
Inhibition of Aβ-peptide aggregation	IC50 = 2.3 μM	IC50 = 0.3 μM	Taniguchi et al. (2005)
Inhibition of tau-protein aggregation	IC50 = 1.9 μM	IC50 = 1.9 μM	Taniguchi et al. (2005)
Inhibition of tau-protein aggregation	$K_i = 3.4\ \mu M$	$K_i = 112\ nM$	Wischik et al. (1996)

Key: AB, azure B; IC50, required drug concentration for 50% inhibition; LPS, lipopolysaccharide; MB, methylene blue.

clarified for the effects of the phenothiazines on the aggregation behavior of neurodegenerative filaments (see below and Table 2).

To our knowledge, azure B has been administered only in rodents (Culo et al., 1991) but not in humans. One of us (R.H.S., 60 kg) experienced that 120 mg azure B dissolved in 30 mL water taken orally led to sensations similar to MB at the same dosage: immediate transient blue discoloration of mucous membranes and teeth, as well as bitter taste (burning but not unpleasant for an adult). Later the urine turned green and then blue, the color intensity being maximal at 12 hours. The tonic invigorating effect described for MB (Cardamatis, 1900; Harvey et al., 2010; Riha et al., 2005) was experienced for azure B at an oral dosage of 2 mg/kg but not at a dosage of 0.5 mg/kg. These observations are consistent with the fact that varying amounts of azure B are present as a contamination in clinically used MB preparations, which has remained unnoticed for more than 100 years.

6. Pleiotropism of MB

There is an amazing number of different molecular targets which have been identified at a molecular level for MB and/or its demethylated metabolites like azure B. The most prominent targets are NO synthases, guanylate cyclase, methemoglobin, monoamino-oxidase A, acetylcholine esterases, and disulfide reductases such as glutathione reductase or dihydrolipoamide dehydrogenase (Buchholz et al., 2008; Harvey et al., 2010; Juffermans et al., 2010). As to the interactions with the flavin-dependent disulfide reductases, MB is not only a (noncompetitive or uncompetitive) inhibitor but also a substrate (Figs. 1 and 2). MB is reduced by the flavoenzyme and the resulting leucoMB is spontaneously oxidized by molecular oxygen (O_2) to give toxic reactive oxygen species (ROS) like superoxide or hydrogen peroxide while MB is formed again. In this way MB is available for the next cycle; it acts — in a functional unit together with flavoenzymes and molecular oxygen — as a recycling catalyst against infectious organisms (Fig. 1). Apart from medical applications, this is the basis for using MB as a disinfectant (Clark et al., 1925), for example as a fungicide in aquariums (see, e.g., www.americanaquarium-products.com).
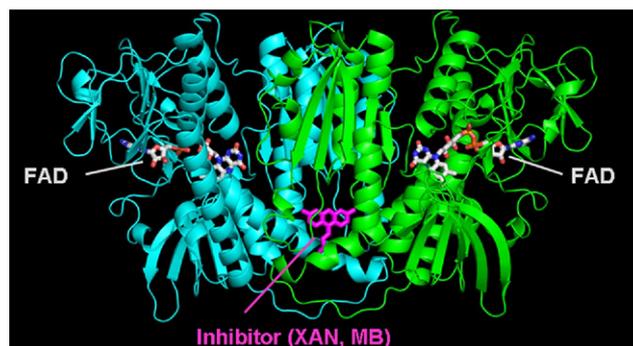

Fig. 2. Crystal structure of the human dimeric enzyme glutathione reductase (GR) with bound tricyclic inhibitor compound. The 2 GR monomers in the dimer are shown with different colors (cyan and green). A xanthene structure is shown as a pink stick model. Methylene blue (MB) derivatives and pyocyanin also bind to this site at the subunit-subunit interface (Schirmer et al., 2008; Fritz-Wolf et al., personal communication). There is probably an additional binding site for phenothiazines acting as reducible substrates. Modified from Savvides and Andrew-Karplus (1996) and Sarma et al. (2003).

7. MB as an analogue of the phenazine compound pyocyanin

An explanation for the pleiotropism of MB could be that it is a thioanalogue of the blue secondary metabolite pyocyanin which acts as a signaling compound and a virulence factor in bacteria (Dietrich et al., 2008). Secondary metabolites (Kossel, 1908) such as caffeine, acetyl salicylate (ACC; aspirin) and most antibiotics have many effects in different organisms but evolution has primed them nevertheless for biospecific interactions (Ahuja et al., 2008; Dietrich et al., 2006, 2008). Human-designed synthetic drugs, in contrast, have not gone through this evolutionary training unless they closely resemble natural compounds.

Recently the pharmacologically active selenoanalogue of MB was synthesized by Herbert Zimmermann (Max Planck Institute, Heidelberg, unpublished). He used a new method whereby selenium — instead of the sulfur (see MB formula in Fig. 1) — is introduced in the very last step of synthesis. The isotopes of seleno-MB, especially the radioactive tracer Se-75-MB, are of interest for systematic studies on the pharmacokinetics and pharmacodynamics of seleno-MB and seleno-azure B (Kühbacher et al., 2009; Kung and Blau, 1980). From a structural perspective, seleno-MB has the additional advantage to allow accurate determination of crystalline enzyme-MB complexes because Se atoms act as anomalous diffraction centers for synchrotron X-ray diffraction (Hendrickson, 1991).

8. Methylene blue for falciparum malaria in children

The revival of MB as an antimalarial drug candidate began in 1995 in 3 biochemical laboratories (Atamna et al., 1996; Färber et al., 1998; Vennerstrom et al., 1995). The major goal of this work is to develop an affordable, available, and accessible therapy for uncomplicated falciparum malaria in children under 5 years of age in Africa. The results of the clinical studies in Nouna, Burkina Faso (directed by Olaf Müller, Boubacar Coulibaly, and Peter Meissner), are promising. Methylene blue-based combination therapy is efficacious and safe even for children with the African form of glucose-6-phosphate dehydrogenase deficiency (G6PD deficiency A^{minus}); the latter condition affects ~15% of the male population in West Africa. As shown in an anthropological study, MB-based therapies are accepted by the communities in spite of the blue discoloration; on the contrary, blue washable spots in clothes or diapers indicate patient compliance to caregivers and health workers (Coulibaly et al., 2009; Müller et al., 2008). The blue color of the urine can also be used as an indicator that the MB-containing drug combination has not been faked. Drug faking is a most serious problem in many developing countries (Müller et al., 2009).

Pharmacokinetics and bioavailability of MB given orally have recently been reinvestigated (Akoachere et al., 2005; Bountogo et al., 2010; Walter-Sack et al., 2009); details are given in the legend of Table 1. MB is active in vitro and in vivo not only against the malaria-causing blood schizonts (Vennerstrom et al., 1995), but also against the gametocytes of *P. falciparum* which are responsible for disease transmission from patients to mosquitoes (Buchholz et al., 2008; Coulibaly et al., 2009). As a therapy, 2 oral doses of 12 mg MB/kg body weight are administered for 3 days, which is in total 72 mg/kg (Zoungrana et al., 2008). Formulations that are suitable for children, a sweet granulate or syrup of MB, have recently been developed (Gut et al., 2008).

Olaf Müller and his team did not observe different effects of MB when using different commercial sources including the 0.1 g MB capsules prepared for us at the University Pharmacy. Since 2007, however, we have had difficulties in obtaining sufficient MB from pharmaceutical companies for the clinical trials in West Africa. In Germany, MB is no longer available even in pediatric emergency rooms (Ludwig and Baethge, 2010). In addition, it was claimed that the available MB preparations were not pure enough, the major contaminants being heavy metals, azure B, and water. By contrast, we regard the prevailing requirements of USP and EP (listed for instance in www.provepharm.com/analysis.php) as appropriate. Taking heavy metal ions as examples, copper and chromium are essential nutrients, and it is interesting to compare their contents in a daily MB dose with their contents in the ingredients of a standard meal. As a conservative physician one is often concerned about the overblown safety requirements of postmodern medicine which too often prevent health- or even life-saving measures.

The explanations given for the shortage of MB were contradictory; the most plausible one was that there are ongoing market rearrangements and price reassessments. Indeed, the price for MB as a raw material — which is now Good Manufacturing Practice (GMP) validated — has recently gone up by a factor of 100. Thus MB will probably no longer serve as an affordable compound for treating malaria as a disease of the poor. In this context it should also be emphasized that MB used to be an ethical preparation which implies that the drug is affordable and available everywhere in sufficient dosages for patients who need it, even when considering that the incidence of malaria exceeds 250 million cases per year. Our title "Lest we forget you" (originally from a poem of Rudyard Kipling, 1887 and later often used as a solemn formula on Remembrance Days) was chosen because we regard MB as a drug that has saved many lives and deserves our continuous respect like other heroes. It is indeed debatable if development, production, and distribution of drugs for malaria and other diseases of the poor should be left to the pharmaceutical industry as there is an enormous need but little demand for these drugs, which means that market rules do not apply.
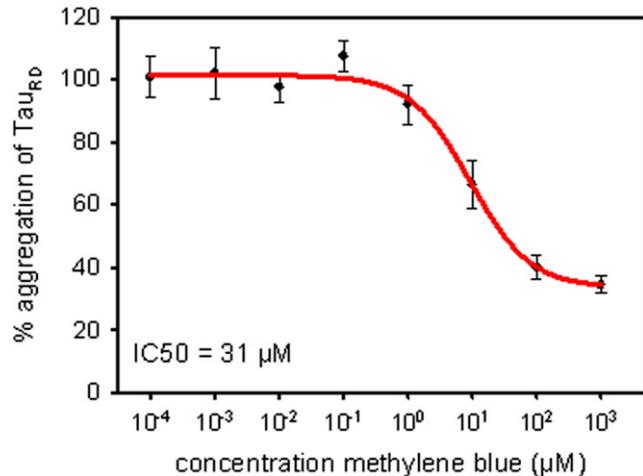
Fig. 3. Inhibition of Tau aggregation by methylene blue (MB) determined by the filter assay. Tau protein (repeat domain, construct K19) was aggregated in the presence of various concentrations of methylene blue. The samples were filtered through a nitrocellulose membrane (Ø 0.45 μm) and the amount of aggregates were detected via Western blotting. For details see Pickhardt et al. (2007), and Bulic et al. (2010).

9. MB for slowing down neurodegenerative diseases?

For the treatment of Alzheimer's disease different approaches are followed. Aβ- or tau-based strategies focus on the modulation at the protein level (e.g., inhibition of proteolytic processing or of phosphorylation) or on the inhibition of protein aggregates. Other potential drugs for Alzheimer's disease (e.g., Dimebon (Biotrend Chemicals), NAP-peptide (NAPVSIPQ; Hölzel Diagnostika)) have different targets (acetylcholine esterase, mitochondrial respi-

ration, cytochrome c-oxidase, NMDA receptor, microtubule stability) and thus may improve the viability of the affected cells. The drug candidates which are presently tested in clinical trials for Alzheimer's disease were reviewed by Neugroschl and Sano (2009, 2010).

During the past decade there has been a growing interest in MB as a potential drug for Alzheimer's disease. This was based on the observations that MB can inhibit the aggregation of tau protein (Wischik et al., 1996) and of Aβ peptides in the low μmol/L range (Chang et al., 2009; Necula et al., 2007; Taniguchi et al., 2005). The potential role of MB as an aggregation inhibitor of Tau can be measured by various assays (Figs. 3 and 4). The reported IC50 values for MB regarding the inhibition of Tau-aggregation show some variation, e.g., 1.9 μM (see Table 2 in Taniguchi et al., 2005) or approximately 3.5 μM (Chang et al., 2009, their Fig. 4). Some of this may be due to different experimental procedures (e.g., filter-based assays vs. fluorescence detection methods). Pelleting assays with polyacrylamide gel electrophoresis as well as electron microscopy reflect not necessarily the full composition of mixed protein aggregates in a given sample because of incomplete separation or absorption. On the other hand the filter method contains little information about the quality (ordered or amorphous) of the detected protein aggregates. Furthermore, the amount of aggregated protein and the activity of aggregation inhibitors are strongly influenced by incubation parameters like the incubation temperature, time, ionic strength and pH of the buffer system (Jeganathan et al., 2008), nature and amount of aggregation-inducing substance (e.g., heparin, arachidonic acid) as well as the properties of the used protein. For example, certain compounds have only 1 bind-
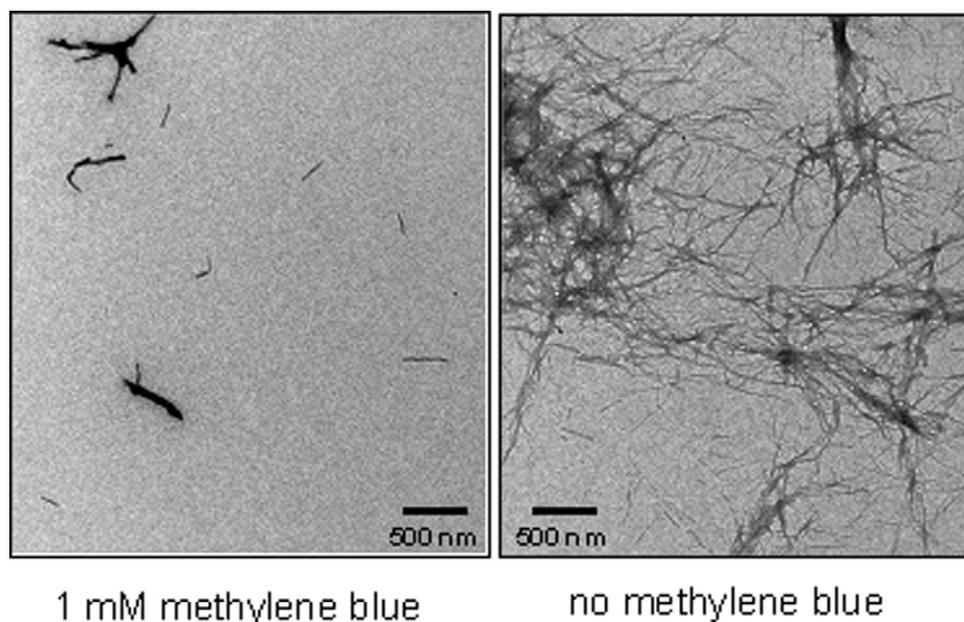

Fig. 4. Electron micrographs of tau filaments (repeat domain) after and before treatment with methylene blue.

ing site in the repeat domain but several in full-length tau, which changes the apparent IC50 concentrations. In summary, these experimental assays may show differences in detail but reflect similar trends (Figs. 3 and 4). The bottom line of Table 2 shows that azure B is a more effective aggregation inhibitor than MB. As a consequence, the proportion of azure B present as an impurity of an MB batch can influence the apparent inhibition constant.

MB received widespread attention following the report by Wischik and colleagues at the International Conference on Alzheimer's Disease in Chicago in 2008 that the formulation Rember[TM] had beneficial effects in clinical trials (Wischik et al., 2008). This claim triggered a wave of comments in scientific journals, blogs, and the public press (for comments by the scientific community see www.alzforum.org). The aggregation inhibitory potential was shown in cell models not only for tau and Aβ, but for other aggregation-prone proteins, notably TDP43 involved in frontotemporal dementias (Arai et al., 2010; Yamashita et al., 2009). It should be noted that even in the case of the dimeric enzyme glutathione reductase, MB or its demethylated metabolites, bind at the interface of the subunits (Fig. 2).

As expected of MB's pleiotropism, other modes of action for MB in Alzheimer's disease were proposed as well. A protective role of MB in senescence and neurodegeneration was postulated to operate via mitochondria and cytochrome c oxidase and thought to be based on the cycling between the oxidized and reduced forms of MB (Atamna and Kumar, 2010; Atamna et al., 2008). It is presently not clear whether a possible therapeutic use of MB in the treatment of neurodegenerative diseases is due to its tau aggregation inhibitory effect, the antioxidant activity by interaction with the electron transport chain of mitochondria, or the binding and modulation of other proteins.

Behavioral and memory studies with rats showed improvement with low doses of MB (a few mg/kg) without other side effects (Riha et al., 2005). This is consistent with the doses that were shown to have no adverse side effects in humans (~300 mg/day, or ~4 mg/kg, Naylor et al., 1986). The positive memory effects are assumed to be due to improved brain glucose and oxygen utilization via stimulation of mitochondrial cytochrome c oxidase. However, adverse effects (e.g., on locomotion) became manifest at higher doses.

Treatment of 3xTg-AD mice showed an improved clearance of soluble Aβ and increased chymotrypsin- and trypsin-like activities of the proteasome, but no effect on tau accumulation, phosphorylation or mislocalization (Medina et al., 2010).

Mixed conclusions were drawn from studies on the interaction between MB and the cellular chaperone system in cell models. MB inhibits the ATPase of hsp70, and one of the consequences is the reduced degradation of toxic poly-Gln tracts and their increased accumulation in the cell (Wang et al., 2010). On the other hand, the same type of hsp70 inhibition is thought to selectively decrease the level of tau protein, thus protecting the cell from toxic accumulations of tau (Jinwal et al., 2009). In zebra fish models of tauopathy, MB showed no effects, in contrast to analogous zebra fish models expressing poly-Gln proteins; here aggregation could be prevented by poly-Gln aggregation inhibitors (van Bebber et al., 2010). In the case of rats, MB was able to reverse the cognitive deficits induced by scopolamine and it acted synergistically with rivastigmine, an acetyl cholinesterase inhibitor used in the treatment of Alzheimer's disease (AD) (Deiana et al., 2009). This result may be explained by the fact that MB acts as a cholinesterase inhibitor as well (Pfaffendorf et al., 1997). Thus MB is expected to interact with the effects of cholinesterase inhibitors presently used in the therapy of Alzheimer's disease. Further information on the physicochemical features and molecular targets in the brain can be found in the reports of Oz et al. (2009, 2011).

One caveat in the interpretation of drug studies is that methylene blue shows a biphasic dose-response relationship with beneficial effects only in an intermediate concentration zone (hormetic effect). This was shown for brain cytochrome oxidase activity and spontaneous locomotor activity in the running wheel test (Bruchey and Gonzales-Lima, 2008). A similar behavior was described for cyanine compounds on tau aggregation in tissue slices (Chang et al., 2009).

Even though the reports on the effects of MB are mixed, there is little doubt that many patients and their caregivers who suffer *now*, are not willing to wait until MB in the form of Rember[TM] might be registered as a drug for use against Alzheimer's disease in 2012 or later (especially since "dosage recommendations" for AD patients are traded on popular web sites). In order to get additional insights into the efficacy of MB it would be interesting to retrospectively study possible neurological effects of MB in patients who have taken MB for the prevention of urinary tract infections (Table 2). If the effects of MB and/or its metabolites on the pathogenesis of AD can be confirmed, these compounds could be administered prophylactically probably for decades. This is illustrated by persons with hereditary methemoglobinemia who take MB for a lifetime in order to prevent the accumulation of the pathological protein methemoglobin in red blood cells (Cawein et al., 1964).

Disclosure statement

The authors declare no conflict of interest.

Acknowledgements

References

Ahuja, E.G., Janning, P., Mentel, M., Graebsch, A., Breinbauer, R., Hiller, W., Costisella, B., Thomashow, L.S., Mavrodi, D.V., Blankenfeldt, W., 2008. PhzA/B catalyzes the formation of the tricycle in phenazine biosynthesis. J. Am. Chem. Soc. 130, 17053–17061.

Akoachere, M., Buchholz, K., Fischer, E., Burhenne, J., Haefeli, W.E., Schirmer, R.H., Becker, K., 2005. In vitro assessment of methylene blue on chloroquine-sensitive and -resistant *Plasmodium falciparum* strains reveals synergistic action with artemisinins. Antimicrob. Agents Chemother. 49, 4592–4597.

Arai, T., Hasegawa, M., Nonoka, T., Kametani, F., Yamashita, M., Hosokawa, M., Niizato, K., Tsuchiya, K., Kobayashi, Z., Ikeda, K., Yoshida, M., Onaya, M., Fujishiro, H., Akiyama, H., 2010. Phosphorylated and cleaved TDP-43 in ALS, FTLD and other neurodegenerative disorders and in cellular models of TDP-43 proteinopathy. Neuropathology 30, 170–181.

Atamna, H., Kumar, R., 2010. Protective role of methylene blue in Alzheimer's disease via mitochondria and cytochrome *c* oxidase. J. Alzheimers Dis. 20, S439–S452.

Atamna, H., Krugliak, M., Shalmiev, G., Deharo, E., Pescarmona, G., Ginsburg, H., 1996. Mode of antimalarial effect of methylene blue and some of its analogues on *Plasmodium falciparum* in culture and their inhibition of *P. vinckei petteri* and *P. yoelii nigeriensis* in vivo. Biochem. Pharmacol. 51, 693–700.

Atamna, H., Nguyen, A., Schultz, C., Boyle, K., Newberry, J., Kato, H., Ames, B.N., 2008. Methylene blue delays cellular senescence and enhances key mitochondrial biochemical pathways. FASEB J. 22, 703–712.

Barcia, J.J., 2007. The Giemsa stain: its history and applications. Int. J. Surg. Pathol. 15, 292–296.

Bodoni, P., 1899. Dell'azione sedativa del bleu di metilene in varie forme di psicosi. Clin. Med. Ital. 21, 217–222.

Bountogo, M., Zoungrana, A., Coulibaly, B., Klose, C., Mansmann, U., Mockenhaupt, F.P., Burhenne, J., Mikus, G., Walter-Sack, I., Schirmer, R.H., Sié, A., Meissner, P., Müller, O., 2010. Efficacy of methylene blue monotherapy in semi-immune adults with uncomplicated falciparum malaria: a controlled trial in Burkina Faso. Trop. Med. Int. Health 15, 713–717.

Bruchey, A.K., Gonzales-Lima, F., 2008. Behavioral, physiological and biochemical hormetic responses to the autoxidizable dye methylene blue. Am. J. Pharmacol. Toxicol. 3, 72–79.

Buchholz, K., Schirmer, R.H., Eubel, J.K., Akoachere, M.B., Dandekar, T., Becker, K., Gromer, S., 2008. Interactions of methylene blue with human disulfide reductases and their orthologues from *Plasmodium falciparum*. Antimicrob. Agents Chemother. 52, 183–191.

Bulic, B., Pickhardt, M., Mandelkow, E.-M., Mandelkow, E., 2010. Tau protein and tau aggregation inhibitors. Neuropharmacology 59, 276–289.

Burrows, G.E., 1984. Methylene blue: effects and disposition in sheep. J. Vet. Pharmacol. Ther. 7, 225–231.

Cajal, S., 1896. El azul de metileno en los centros nerviosos. Rev. Trimest. Microgr. 1, 151–203.

Cardamatis, A., 1900. Methylene blue in grave malaria cachexia. JAMA 34, 1408–1409.

Cawein, M., Behlen, C.H. 2nd, Lappat, E.J., Cohn, J.E., 1964. Hereditary diaphorase deficiency and methemoglobinemia. Arch. Intern. Med. 113, 578–585.

Chang, E., Congdon, E.E., Honson, N.S., Duff, K.E., Kuret, J., 2009. Structure-activity relationship of cyanine tau aggregation inhibitors. J. Med. Chem. 52, 3539–3547.

Clark, W.M., Cohen, B., Gibbs, H.D., 1925. Studies on oxidation-reduction. VIII. Methylene blue. U. S. Pub Health Rep. 40, 1131–1201.

Coulibaly, B., Zoungrana, A., Mockenhaupt, F.P., Schirmer, R.H., Klose, C., Mansmann, U., Meissner, P.E., Müller, O., 2009. Strong gametocytocidal effect of methylene blue-based combination therapy against falciparum malaria: a randomised controlled trial. PLoS One 4, e5318.

Culo, F., Sabolović, D., Somogyi, L., Marusić, M., Berbiguier, N., Galey, L., (1991). Anti-tumoral and anti-inflammatory effects of biological stains. Agents Actions 34, 424–428.

Deiana, S., Harrington, C.R., Wischik, C.M., Riedel, G., 2009. Methylthioninium chloride reverses cognitive deficits induced by scopolamine: comparison with rivastigmine. Psychopharmacology (Berl) 202, 53–65.

Dietrich, L.E., Price-Whelan, A., Petersen, A., Whiteley, M., Newman, D.K., 2006. The phenazine pyocyanin is a terminal signalling factor in the quorum sensing network of *Pseudomonas aeruginosa*. Mol. Microbiol. 61, 1308–1321.

Dietrich, L.E., Teal, T.K., Price-Whelan, A., Newman, D.K., 2008. Redox-active antibiotics control gene expression and community behavior in divergent bacteria. Science 321, 1203–1206.

Ehrlich, P., 1886. Über die Methylenblaureaktion der lebenden Nervensubstanz. Dtsch. Med. Wochenschr. 12, 49–52.

Ehrlich, P., Leppmann, A., 1890. Über Schmerzstillende Wirkung des Methylenblau. Dtsch. Med. Wochenschr. 16, 493–494.

Färber, P.M., Arscott, L.D., Williams, C.H., Jr., Becker, K., Schirmer, R.H., 1998. Recombinant Plasmodium falciparum glutathione reductase is inhibited by the antimalarial dye methylene blue. FEBS Lett. 422, 311–314.

Fleischer, B., 2004. Editorial: 100 years ago: Giemsa's solution for staining of plasmodia. Trop. Med. Int. Health 9, 755–756.

Garcia-Lopez, P., Garcia-Marin, V., Freire, M., 2007. The discovery of dendritic spines by Cajal in 1888 and its relevance in the present neuroscience. Prog. Neurobiol. 83, 110–130.

Gaudette, N.F., Lodge, J.W., 2005. Determination of methylene blue and leucomethylene blue in male and female Fischer 344 rat urine and B6C3F1 mouse urine. J. Anal. Toxicol. 29, 28–33.

Gura, T., 2008. Hope in Alzheimer's fight emerges from unexpected places. Nat. Med. 14, 894.

Gut, F., Schiek, W., Haefeli, W.E., Walter-Sack, I., Burhenne, J., 2008. Cation exchange resins as pharmaceutical carriers for methylene blue: binding and release. Eur. J. Pharm. Biopharm. 69, 582–587.

Guttmann, P., Ehrlich, P., 1891. Über die Wirkung des Methylenblau bei Malaria. Berlin. Klin. Woch. 28, 953–956.

Harvey, B.H., Duvenhage, I., Viljoen, F., Scheepers, N., Malan, S.F., Wegener, G., Brink, C.B., Petzer, J.P., 2010. Role of monoamine oxidase, nitric oxide synthase and regional brain monoamines in the antidepressant-like effects of methylene blue and selected structural analogues. Biochem. Pharmacol. 80, 1580–1591.

Hattori, M., Sugino, E., Minoura, K., In, Y., Sumida, M., Taniguchi, T., Tomoo, K., Ishida, T., 2008. Different inhibitory response of cyanidin and methylene blue for filament formation of tau microtubule-binding domain. Biochem. Biophys. Res. Commun. 374, 158–163.

Hendrickson, W.A., 1991. Determination of macromolecular structures from anomalous diffraction of synchrotron radiation. Science 254, 51–58.

Jeganathan, S., von Bergen, M., Mandelkow, E.-M., Mandelkow, E., 2008. The natively unfolded character of tau and its aggregation to Alzheimer-like paired helical filaments. Biochemistry 47, 10526–10539.

Jinwal, U.K., Miyata, Y., Koren, J. 3rd, Jones, J.R., Trotter, J.H., Chang, L., O'Leary, J., Morgan, D., Lee, D.C., Shults, C.L., Rousaki, A.,

Weeber, E.J., Zuiderweg, E.R., Gestwicki, J.E., Dickey, C.A., 2009. Chemical manipulation of hsp70 ATPase activity regulates tau stability. Neuroscience 29, 12079–12088.

Juffermans, N.P., Vervloet, M.G., Daemen-Gubbels, C.R.G., Binnekade, J.M., de Jong, M., Groeneveld, A.B.J., 2010. A dose-finding study of methylene blue to inhibit nitric oxide actions in the hemodynamics of human septic shock. Nitric Oxide 22, 275–280.

Khavandi, A., Whitaker, J., Gonna, H., 2008. Serotonin toxicity precipitated by concomitant use of citalopram and methylene blue. Med. J. Aust. 189, 534–535.

Kossel, A., 1908. Die Probleme der Biochemie. Universitäts-Buchdruckerei Heidelberg.

Kristiansen, J.E., 1989. Dyes, antipsychotic drugs, and antimicrobial activity. Fragments of a development, with special reference to the influence of Paul Ehrlich. Dan. Med. Bull. 36, 178–185.

Kühbacher, M., Bartel, J., Hoppe, B., Alber, D., Bukalis, G., Bräuer, A.U., Behne, D., Kyriakopoulos, A., 2009. The brain selenoproteome: priorities in the hierarchy and different levels of selenium homeostasis in the brain of selenium-deficient rats. J. Neurochem. 110, 133–142.

Kung, H.F., Blau, M., 1980. Regional intracellular pH shift: a proposed new mechanism for radiopharmaceutical uptake in brain and other tissues. J. Nucl. Med. 21, 147–152.

Kupfer, A., Aeschlimann, C., Wermuth, B., Cerny, T., 1994. Prophylaxis and reversal of ifosfamid encephalopathy with methylene-blue. Lancet 343, 763–764.

Ludwig, S., Baethge, C., 2010. Lebensbedrohliche Kreislaufreaktionen auf Toluidinblau. Deutsches Ärzteblatt 107, C1308.

MacArthur, D., 1964, and 2001. Reminiscences. 2nd Edition. Bluejacket Books, Annapolis, MD.

Medina, D.X., Caccamo, A., Oddo, S., 2010. Methylene blue reduces Abeta levels and rescues early cognitive deficit by increasing proteasome activity. Brain Pathol., in press.

Mentes, B.B., Akin, M., Leventoglu, S., Gultekin, F.A., Oguz, M., 2004. Intradermal methylene blue injection for the treatment of intractable idiopathic pruritus ani: results of 30 cases. Tech. Coloproctol. 8, 11–14.

Müller, O., Meissner, P., Schirmer, R.H., 2008. Methylenblau in der Malariatherapie. Eine Alternative für Afrika? Flugmed. Tropenmed. Reisemed. (FTR) 14, 33–35.

Müller, O., Sié, A., Meissner, P., Schirmer, R.H., Kouyaté, B., 2009. Artemisinin resistance on the Thai-Cambodian border. Lancet 374, 1418–1419.

Naylor, G.J., Martin, B., Hopwood, S.E., Watson, Y., 1986. A two-year double-blind crossover trial of the prophylactic effect of methylene blue in manic depressive psychosis. Biol. Psychiatry 21, 915–920.

Necula, M., Breydo, L., Milton, S., Kayed, R., van der Veer, W.E., Tone, P., Glabe, C.G., 2007. Methylene blue inhibits amyloid Abeta oligomerization by promoting fibrillization. Biochemistry 46, 8850–8860.

Neugroschl, J., Sano, M., 2009. An update on treatment and prevention strategies for Alzheimer's disease. Curr. Neurol. Neurosci. Rep. 9, 368–376.

Neugroschl, J., Sano, M., 2010. Current treatment and recent clinical research in Alzheimer's disease. Mt Sinai. J. Med. 77, 3–16.

O'Leary, J.L., Petty, J., Harris, A.B., Inukai, J., 1968. Supravital staining of mammalian brain with intra-arterial methylene blue followed by pressurized oxygen. Stain Technol. 43, 197–201.

Oz, M., Lorke, D.E., Hasan, M., Petroianu, G.A., 2011. Cellular and molecular actions of methylene blue in the nervous system. Med. Res. Rev. 31, 93–117.

Oz, M., Lorke, D.E., Petroianu, G.A., 2009. Methylene blue and Alzheimer's disease. Biochem. Pharmacol. 78, 927–932.

Pelgrims, J., De Vos, F., Van den Brande, J., Schrijvers, D., Prove, A., Vermorken, J.B., 2000. Methylene blue in the treatment and prevention of ifosfamide-induced encephalopathy: report of 12 cases and a review of the literature. Br. J. Cancer 82, 291–294.

Peter, C., Hongwan, D., Kupfer, A., Lauterburg, B.H., 2000. Pharmacokinetics and organ distribution of intravenous and oral methylene blue. Eur. J. Clin. Pharmacol. 56, 247–250.

Pfaffendorf, M., Bruning, T.A., Batnik, H.D., van Zwieten, P.A., 1997. The interaction between methylene blue and the cholinergic system. Br. J. Pharmacol. 122, 95–98.

Pickhardt, M., Larbig, G., Khlistunova, I., Coksezen, A., Meyer, B., Mandelkow, E.M., Schmidt, B., Mandelkow, E., 2007. Phenylthiazolyl-hydrazide and its derivatives are potent inhibitors of tau aggregation and toxicity in vitro and in cells. Biochemistry 46, 10016–10023.

Ramsay, R.R., Dunford, C., Gillman, P.K., 2007. Methylene blue and serotonin toxicity: inhibition of monoamine oxidase A (MAO A) confirms a theoretical prediction. Br. J. Pharmacol. 152, 946–951.

Riha, P.D., Bruchey, A.K., Echevarria, D.J., Gonzalez-Lima, F., 2005. Memory facilitation by methylene blue: dose-dependent effect on behavior and brain oxygen consumption. Eur. J. Pharmacol. 511, 151–158.

Sarma, G.N., Savvides, S.N., Becker, K., Schirmer, M., Schirmer, R.H., Karplus, P.A., 2003. Glutathione reductase of the malarial parasite *Plasmodium falciparum*: Crystal structure and inhibitor development. J. Mol. Biol. 328, 893–907.

Savvides, S.N., Karplus, P.A., 1996. Kinetics and crystallographic analysis of human glutathione reductase in complex with a xanthene inhibitor. J. Biol. Chem. 271, 8101–8107.

Schirmer, R.H., Adler, H., Zappe, H.A., Gromer, S., Becker, K., Coulibaly, B., Meissner, P. 2008. Disulfide reductases as drug targets: methylene blue combination therapies for falciparum malaria in African children. in: Flavins and Flavoproteins, Prensas Universitarias de Zaragoza, Zaragoza, Spain, 481–486.

Shanmugam, G., 2005. Vasoplegic syndrome–the role of methylene blue. Eur. J. Cardiothorac. Surg. 28, 705–710.

Sutherland, A.D., Faragher, I.G., Frizelle, F.A., 2009. Intradermal injection of methylene blue for the treatment of refractory pruritus ani. Colorectal Dis. 11, 282–287.

Tan, C.D., Rodriguez, E.R., 2010. Blue dye, green heart. Cardiovasc. Pathol. 19, 125–126.

Taniguchi, S., Suzuki, N., Masuda, M., Hisanaga, S., Iwatsubo, T., Goedert, M., Hasegawa, M., 2005. Inhibition of heparin-induced tau filament formation by phenothiazines, polyphenols, and porphyrins. J. Biol. Chem. 280, 7614–7623.

van Bebber, F., Paquet, D., Hruscha, A., Schmid, B., Haass, C., 2010. Methylene blue fails to inhibit Tau and polyglutamine protein dependent toxicity in zebrafish. Neurobiol. Dis. 39, 265–271.

Van der Horst, C., Stuebinger, H., Seif, C., Melchior, D., Martinez-Portillo, F.J., Juenemann, K.P., 2003. Priapism - etiology, pathophysiology and management. Int. Braz. J. Urol. 29, 391–400.

Vennerstrom, J.L., Makler, M.T., Angerhofer, C.K., Williams, J.A., 1995. Antimalarial dyes revisited: xanthenes, azines, oxazines, and thiazines. Antimicrob. Agents Chemother. 39, 2671–2677.

Wainwright, M., Amaral, L., 2005. The phenothiazinium chromophore and the evolution of antimalarial drugs. Trop. Med. Int. Health 10, 501–511.

Walter-Sack, I., Rengelshausen, J., Oberwittler, H., Burhenne, J., Müller, O., Meissner, P., Mikus, G., 2009. High absolute bioavailability of methylene blue given as an aqueous oral formulation. Eur. J. Clin. Pharmacol. 65, 179–189.

Wang, A.M., Morishima, Y., Clapp, K.M., Peng, H.M., Pratt, W.B., Gestwicki, J.E., Osawa, Y., Lieberman, A.P., 2010. Inhibition of hsp70 by methylene blue affects signaling protein function and ubiquitination and modulates polyglutamine protein degradation. J. Biol. Chem. 285, 15714–15723.

Warth, A., Goeppert, B., Bopp, C., Schirmacher, P., Flechtenmacher, C., Burhenne, J., 2009. Turquoise to dark green organs at autopsy. Virchows Arch. 454, 341–344.

Wischik, C.M., Bentham, P., Wischik, D.J., Seng, K.M., 2008. Tau aggregation inhibitor (TAI) therapy with Rember™ arrests disease progression in mild and moderate Alzheimer's disease over 50 weeks. Alzheimers Dement. 4, T167.

Wischik, C.M., Edwards, P.C., Lai, R.Y., Roth, M., Harrington, C.R., 1996. Selective inhibition of Alzheimer disease-like tau aggregation by phenothiazines. Proc. Natl. Acad. Sci. U. S. A. 93, 11213–11218.

Wolloch, Y., Dintsman, M., 1979. A simple and effective method of treatment for intractable pruritus ani. Am. J. Proctol. Gastroenterol. Colon Rect. Surg. 30, 34–36.

Yamashita, M., Nonaka, T., Arai, T., Kametani, F., Buchman, V.L., Ninkina, N., Bachurin, S.O., Akiyama, H., Goedert, M., Hasegawa, M., 2009. Methylene blue and dimebon inhibit aggregation of TDP-43 in cellular models. FEBS Lett. 583, 2419–2424.

Zoungrana, A., Coulibaly, B., Sié, A., Walter-Sack, I., Mockenhaupt, F.P., Kouyate, B., Schirmer, R.H., Klose, C., Mansmann, U., Meissner, P., Müller, O., 2008. Safety and efficacy of methylene blue combined with artesunate or amodiaquine for uncomplicated falciparum malaria: a randomized controlled trial from Burkina Faso. PLoS One 3, e1630.