METHYLENE BLUE ADMINISTRATION FAILS TO CONFER NEUROPROTECTION IN TWO AMYOTROPHIC LATERAL SCLEROSIS MOUSE MODELS

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Abstract—Approximately 20% cases of familial amyotrophic lateral sclerosis (ALS) are caused by mutations in the gene encoding Cu/Zn superoxide dismutase (SOD1). Recent studies have shown that methylene blue (MB) was effective in conferring protection in several neurological disorders. MB was found to improve mitochondrial function, to reduce reactive oxygen species, to clear aggregates of toxic proteins, and to act as a nitric oxide synthase inhibitor. These pleiotropic effects of relevance to ALS pathogenesis led us to test MB in two models of ALS, SOD1G93A mice and TDP-43G348C transgenic mice. Intraperitoneal administration of MB at two different doses was initiated at the beginning of disease onset, at 90 days of age in SOD1G93A and at 6 months of age in TDP-43G348C mice. Despite its established neuroprotective properties, MB failed to confer protection in both mouse models of ALS. The lifespan of SOD1G93A mice was not affected by MB treatment. The declines in motor function, reflex score, and body weight of SOD1G93A mice remained unchanged. MB treatment had no effect on motor neuron loss and aggregation or misfolding of SOD1. A combination of MB with lithium also failed to provide benefits in SOD1G93A mice. In TDP-43G348C mice, MB failed to improve motor function. Cytosolic translocation of TDP-43, ubiquitination and inflammation remained also unchanged after MB treatment of TDP-43G348C mice. © 2012 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: methylene blue, superoxide dismutase, TDP-43, ALS, neuroinflammation.

Amyotrophic lateral sclerosis (ALS) is the most common adult-onset motor neuron disease, leading to progressive paralysis and death. Ninety percent of the cases are sporadic (sALS), and the remaining are familial (fALS), but the two forms are clinically and pathologically distinguishable. Twenty percent of the familial cases are related to mutations in the Cu/Zn superoxide dismutase gene (SOD1) and although the mechanisms leading to pathology remain unclear (Boillée et al., 2006). Transgenic mice expressing several SOD1 mutants have been widely used to understand the ALS pathology because they were found to develop motor neuron disease very similar to the human disease (Turner and Talbot, 2008). Various hypotheses have been proposed to explain the toxicity of SOD1 mutant protein including aggregation (reviewed in Chattopadhyay and Valentine, 2009; Ticozzi et al., 2010), oxidative stress (reviewed in Barber and Shaw, 2010) and mitochondrial dysfunction (reviewed in Pizzuti and Petrucci, 2011), excitotoxicity (reviewed in Boogaert et al., 2010), and more recently RNA processing through TDP-43 and FUS/TLS abnormalities (reviewed in Baumer et al., 2010; Lagier-Tourenne et al., 2010). Although pathological pathways leading to ALS seem to differ between SOD1 and TDP-43 cases, a common hallmark resides in toxic protein aggregation (Chattopadhyay and Valentine, 2009; Johnson et al., 2009).

Although numerous compounds have been tested to treat ALS, most of them were proven ineffective, except riluzole, which slightly prolongs survival of patients (Miller et al., 2007). Methylene blue (MB), a monoamine oxidase inhibitor, has been used for more than a century to treat several diseases and infections. It acts as an inhibitor of NO synthase, whose upregulation occurs in motoneurons and reactive astrocytes of ALS patients (Anneser et al., 2001; Sasaki et al., 2001), as well as in SOD1G93A mice (Cha et al., 1998; Almer et al., 1999; Sasaki et al., 2002). MB can also improve mitochondrial function and be an effective electron carrier, thus acting on reactive oxygen species (Atamna et al., 2008; Wen et al., 2011), which can also be linked to ALS. Increasing evidence shows that methylene blue has strong neuroprotective effects in a growing list of neurological disorders, including Alzheimer’s disease (Wischik et al., 1996; Atamna and Kumar, 2010; Medina et al., 2011), Parkinson’s disease (Wen et al., 2011), cerebral ischemia (Wiklund et al., 2007; Miclescu et al., 2010; Wen et al., 2011), amnesia (Riha et al., 2011), and bipolar disorder (Naylor et al., 1981; Narasapu and Naylor, 1983; Ergul and Caglayan, 1997). Furthermore, MB has already been proposed as a potential treatment for ALS, as it clears TDP-43 aggregates in cellular models (Yamashita et al., 2009). Moreover, MB has been shown to prolong survival of normal mice and rats (National Toxicology Program, 2008). The latter may also be relevant to ALS, as a premature senescence of motoneurons may be a cause of ALS (McComas et al., 1973).

During the last years, lithium has also raised a lot of attention as a potential treatment for ALS. Positive results were reported from mouse studies and a clinical trial (Shin et al., 2007; Feng et al., 2008; Fornai et al., 2008), but this was followed more recently by negative results with mice (Gill et al., 2009; Pizzarese et al., 2009) and humans (Aggarwal et al., 2010; Chio et al., 2010). Various hypoth-
esizes were formulated to explain those divergent outcomes. However it seems that paradigms combining lithium treatment with other compounds often result in improvement of the disease (Shin et al., 2007; Feng et al., 2008). Besides, in an attempt to diminish seizures in an epilepsy model in mice, a combination of lithium with MB produced a significant decrease of seizures when compared with lithium alone (Bahremand et al., 2010). Thus, it may be relevant to assess the synergic potential of lithium with MB.

Here, we evaluated the efficiency of MB alone or in combination with lithium in mouse SOD1G93A, a well-established and characterized model of ALS. Because there is growing evidence that sporadic ALS cases with TDP-43 abnormalities have a different etiology than familial ALS caused by SOD1 (Neumann et al., 2006; Orrell, 2010), we also tested the effectiveness of MB in the new TDP-43G348C model of ALS (Swarup et al., 2011). These TDP-43 transgenic mice recapitulate well pathological hallmarks of ALS/FTD, making it a good model to further validate the efficiency of MB. In contrast to many other neurological disorders, we report that administration of MB, alone or in combination with lithium, conferred no protection in ALS pathogenesis caused by mutant SOD1 or by mutant TDP-43.

**EXPERIMENTAL PROCEDURES**

**Animals**

SOD1G93A mice [stock number 002726] were acquired from the Jackson Laboratory (Bar Harbor, ME, USA) and enriched in C57BL/6JHsd strain (n=20). SOD1G93A mice were genotyped in accordance with Jackson Laboratory protocols. SOD1G93A mice were injected at the beginning of the symptomatic stage (90 days) every 2 days until their death. TDP-43G348C mice were generated in C3B6 background (described in Swarup et al., 2011) and backcrossed in C57BL/6 background for at least 10 generations. TDP-43G348C mice were injected at the beginning of the symptomatic stage (6 months) every 2 days until 12 months. Methylen blue (Sigma, St-Louis, MO, USA) was dissolved in 0.9% saline, and mice were given 1 mg/kg (SOD1G93A and TDP-43G348C mice) or 10 mg/kg (SOD1G93A mice) intraperitoneally. Lithium (Sigma) was dissolved in 0.9% saline and injected intraperitoneally at a dose of 10 mg/kg (SOD1G93A mice). The use and maintenance of the mice described in this article were performed in accordance to the Guide of Care and Use of Experimental Animals of the Canadian Council on Animal Care. The number of animals and their suffering has been minimized.

**Analysis of disease progression**

Measurements of body weight, hind limb reflex, and rotarod performance were used to score the clinical effects of SOD1G93A mice. The extensibility and postural reflex of the hind limbs when mice were held up with their tails were scored as described previously (Urushitani et al., 2006). The SOD1G93A reflex score and body weight were measured every 2 days, beginning at 90 days. Scoring was performed in a blind manner by animal technicians who had no information about the genotype but had experience in grading SOD1 mice paralysis. Analysis of TDP-43G348C and SOD1G93A mice disease progression was performed with an accelerated rotarod, starting at 4 rpm with a 0.25 rpm/s acceleration, and time was noted when the mice fell off the roll. Three trials were done per animal, and the mean value was calculated for statistics and graphs. Rotarod tests for SOD1G93A and TDP-43G348C mice were performed once a week.

**Tissue collection and immunohistochemical analyses**

Mice were anesthetized and transcardially perfused with NaCl 0.9% and fixed with 4% paraformaldehyde. Dissected spinal cord tissues were postfixed for 24 h in 4% paraformaldehyde and equilibrated in a solution of PBS-sucrose (20%) for 48 h. Spinal cord tissues were cut in 25 μm thick sections with a Leica frozen microtome and kept in a cryoprotective solution at −20 °C. For SOD1G93A mice, sections were incubated with anti-misfolded SOD1 antibody A5C3 (Gros-Louis et al., 2010) (Medimabs, Montreal, Canada), stained with the fluorophore-coupled secondary antibody Alexa-488 (Invitrogen, Carlsbad, CA, USA), and counterstained with DAPI. For TDP-43G348C mice, sections were incubated with monoclonal anti-human TDP-43 (Abnova, Taipei, Taiwan), anti-ubiquitin (Abcam, Cambridge, MA, USA), anti-GFAP (formerly Chemicon—Millipore, Billerica, MA, USA), or anti-Iba1 (Wako, Osaka, Japan) antibodies stained with the fluorophore-coupled secondary antibody Alexa-647 (Invitrogen). Dissected dorsal root ganglia (DRG) were postfixed in a solution of 3% glutaraldehyde for a period of 48 h, washed in PBS, treated with 1% osmium tetroxide for 2 h, and dehydrated through graded alcohol solutions. Before Epon plastic embedding, DRG were further dissected to ensure that all ventral root (VR) axons would be sampled at a distance of 3 mm from the DRG cell body. Semi-thin cross sections (1 μm) were stained with Toluidine Blue, rinsed, and coverslipped. To quantify the immunoreactivity (IR) score on immunohistochemistries, we measured the optical densities of each staining with ImageJ software (NIH). For A5C3, Iba1, and GFAP immunofluorescences, the whole signal intensity was read. To quantify cytoplasmic TDP-43, we surrounded all motoneurons in the spinal cord slices based on their morphology and removed from selection the nucleus for the cytoplasmic measurement. We then read the whole cell intensity and divided the cytoplasm on the whole cell signal to obtain a percentage of cytoplasmic TDP-43 translocalization. For ubiquitin quantification, we measured the amount of ubiquitin-positive inclusions exclusively in the cytoplasmic part of motoneurons.

**Western blotting**

Total spinal cord lysates from SOD1G93A transgenic mice and from non-transgenic littermates were prepared by homogenization in 1 mL of TNG-T buffer consisting of 50 mM Tris–HCl, pH 7.4; 150 mM NaCl; 10% glycerol; 1% Triton X-100; protease inhibitor mixture (Roche, Indianapolis, IN, USA). After homogenization, the tissue suspension was centrifuged for 15 min at 1000 g at 4 °C. The supernatant (soluble fraction) and the pellet (insoluble fraction) were denatured in the sampling buffer containing 2-mercaptoethanol and SDS with boiling. After migration on standard SDS-PAGE gels, the proteins were blotted on PVDF (PerkinElmer, Waltham, MA, USA) membrane. The membranes were labeled with commercially available anti-SOD1 (Stressgen, Ann Arbor, MI, USA). The amount of loaded proteins was verified by staining the same membranes and incubating with anti-actin antibody (Chemicon). The blots were detected using IgG conjugated with peroxidase and chemiluminescent assay (PerkinElmer). The Western bands were scanned and analyzed by densitometry using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

**Statistical analyses**

Data were analyzed using Prism 5.0 software (GraphPad Software, LaJolla, CA, USA). Behavioral data were computed by performing two-way ANOVAs (except when specified) followed by Bonferroni post-tests and survival data using Mantel-Cox log-rank tests. VR axon counts were compared using two-tailed Student’s t-tests. Data are expressed as mean±SEM; P<0.05 was considered statistically significant. One-way ANOVA followed by Bonfer-
roni post-test was performed on A5C3 IR scores (SOD1\textsuperscript{G93A} mice) and unpaired \textit{t}-tests were performed on all other IR scores (TDP-43\textsuperscript{G348C} mice).

**RESULTS**

Methylene blue treatment, alone or in combination with lithium, did not affect lifespan or phenotype of SOD1\textsuperscript{G93A} mice

Following injection of SOD1\textsuperscript{G93A} mice with methylene blue, lithium, or both drugs starting at 90 days of age, we analyzed the lifespan of transgenic mice (Fig. 1A). Mice treated with methylene blue (1 mg/kg) had a lifespan of 141 days, those treated with a combination of methylene blue (1 mg/kg) and lithium (10 mg/kg) had a lifespan of 141.5, those treated with lithium (10 mg/kg) alone lived 139 days; whereas saline injected mice had a median survival of 141 days. The difference was not significant between any of the groups ($P=0.7337$; $n=10$, $n=9$, and $n=18$, respectively). Moreover, analysis of phenotype by reflex scores and rotarod scores showed no difference between any of the groups ($P=0.3173$ and $P=0.8366$, respectively) (Fig. 1B, C). Correspondingly, body weight measures revealed no difference in disease progression of transgenic mice following any of the treatment ($P=0.5070$) (Fig. 1D). To make sure that the dose of methylene blue was sufficient to induce an effect, we also treated SOD1\textsuperscript{G93A} mice with a 10 mg/kg dose of methylene blue under the same parameters (data not shown). The treated animal had a lifespan of 137.5 days compared with 141 days for the saline treated mice, and again this difference was not statistically significant ($P=0.3268$, $n=8$ and $n=18$, respectively).

MB administration had no effect on motor function of TDP-43\textsuperscript{G348C} mice

As expected, TDP-43\textsuperscript{G348C} transgenic mice performed less well on rotarod assessment than wild-type (WT) mice during all the tested period ($P<0.0001$, $n=9$ and $n=6$).

![Fig. 1. Treatment with MB, lithium, or both drugs simultaneously does not affect survival or phenotypes of SOD1\textsuperscript{G93A} mice.](image-url)

- (A) Survival: Kaplan–Meier survival curve shows that saline treated transgenic mice Sod1\textsuperscript{G93A} ($n=19$) had a mean survival of 141.0 d, whereas MB ($n=10$), Li ($n=9$), or MB+Li ($n=8$) treated mice lived for 141.0, 139.0, and 141.5 d, respectively. Log-rank test shows no significance ($P=0.7337$).
- (B) Reflex score: two-way ANOVA revealed that the treatment had no effect on the reflex score throughout the time points ($P=0.3173$). (C) Rotarod score: Regardless of the treatment, the performance of the transgenic mice (gray) was similar on the rotarod until the end of the measurements ($P=0.8366$). Wild-type (WT) mice almost always perform at maximum value (2 min) irrespective of the treatment. (D) Body weight. WT mice consistently gain weight with age unrelatedly of the treatment, whereas transgenic mice lose weight as the paralysis progresses. However, the progressive decrease in the bodyweight of transgenic mice after the onset was comparable between all the groups ($P=0.5070$). For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.
Fig. 2. MB did not improve motor function of TDP-43G348C mice. Rotarod tests were assessed in three consecutive trials every week and the mean value is plotted. WT mice performs significantly better than TDP-43G348C transgenic mice (\( P = 0.0001, n = 6 \) and \( n = 5 \), respectively). There was no difference between MB treated transgenic mice and saline treated transgenic mice neither in any of the time points nor in the overall curve (\( P = 0.8801, n = 4 \) and \( n = 5 \), respectively). There was no difference between MB treated transgenic (\( \pm \)) animals versus MB-treated transgenic (\( \pm \)) animals (\( P = 0.8801, n = 5 \) and \( n = 4 \), respectively).

**Cellular hallmarks of ALS were unchanged after MB treatment**

Misfolded SOD1 is a pathological hallmark of ALS, even in sporadic cases (Bosco et al., 2010; Forsberg et al., 2010). For this reason, we examined its presence with a monoclonal antibody (A5C3) that is specific to misfolded SOD1 (Fig. 3A). However, following any of the treatments, the amount of misfolded SOD1 found in the spinal cord of SOD1G93A mice was not diminished. Comparison of IR scores showed no difference in A5C3 signal for all treatments (\( P = 0.4593, n = 3 \) (Fig. 3C)). In TDP-43 transgenic mice as well as in ALS patients, cytosolic TDP-43 translocation is a well-known pathological hallmark of ALS (Arai et al., 2006; Neumann et al., 2006; Swarup et al., 2011). Monoclonal antibody against human TDP-43 revealed no observable difference in the amount of translocated TDP-43 between treated transgenic animal versus transgenic controls (Fig. 3B), and this was confirmed by quantification of the percentage of TDP-43 found in cytoplasm (\( P = 0.8662, n = 3 \), see figure legend for details) (Fig. 3D). Similarly, the extent of ubiquitination in the amount of translocated TDP-43 between treated transgenic animal versus transgenic controls (Fig. 3B) and their quantification (respectively: \( P = 0.9644, n = 4 \); \( P = 0.8263, n = 5 \)) (Fig. 3D).

**MB does not affect the number of surviving axons in the dorsal root ganglia of SOD1G93A mice**

Axonal degeneration correlates with the disease severity in SOD1 mutant mice (Gurney et al., 1994; Wong et al., 1995; Bruijn et al., 1997). Therefore, we performed transversal sections of the dorsal root ganglia and assessed the axonal degeneration in the VR (Fig. 4A). While the non-transgenic mice had nearly a thousand motoneurons remaining (Fig. 4B). However, there was no difference in the number of axons of mice treated with MB, lithium, or both drugs together (MB: 357, Li: 357.5, MB+Li: 346.5, Saline: 378.0; \( P = 0.5157 \)).

**Accumulation of insoluble SOD1 is not diminished by MB treatment**

It has been proposed that MB may play a role in clearance of a variety of toxic insoluble aggregates. In fact, it was demonstrated that MB can inhibit aggregate formation of a variety of proteins (Wischik et al., 1996; Taniguchi et al., 2005; Yamashita et al., 2009). To verify this hypothesis in SOD1G93A mice, we compared the ratio of soluble versus insoluble fractions of SOD1 in the spinal cord of SOD1G93A mice, treated or not (Fig. 5). In the non-transgenic (WT) mice we mostly detected soluble Sod1, whereas transgenic SOD1G93A mice had a considerable amount insoluble SOD1, as expected. However, the extent of aggregation was not lessened by MB treatment, lithium, or both treatments together.

**DISCUSSION**

Here we demonstrate that MB, Li, or both drugs administered jointly had no effect on the disease caused by SOD1G93A in mice. MB also failed to alleviate disease caused by TDP-43G348C in mice. Our analyses demonstrated that MB was unable to attenuate pathological hallmarks of ALS in either SOD1G93A mice or TDP-43G348C mice.

An earlier study concluded of MB ineffectiveness in conferring protection by oral administration at a dose of 25 mg/kg, which is much higher than the safe working dose already used in mice and in humans for other pathologies (Lougheed and Turnbull, 2011). This could have led to a toxic outcome, masking potential beneficial effects of MB. In another paradigm, a low dose of MB was efficient, whereas the high dose was not able to induce any beneficial effects (Callaway et al., 2004). Actually, toxicity of MB generally occurs at a dosage above 6–12.5 mg/kg (depending on the studies), and the recommended dosage is 1–2 mg/kg (Wiklund et al., 2007; National Toxicology Program, 2008). In this study we treated all mice with a dose of 1 mg/kg of MB, which is within the mean working dose range used in most other paradigms in mice and humans when injected (0.5–2.5 mg/kg) (Clifton and Leikin, 2003; Callaway et al., 2004; Wiklund et al., 2007; Miclescu et al., 2010; Wen et al., 2011). Moreover, it has been demonstrated that MB crosses easily the blood–brain barrier, resulting in high concentrations in the CNS even at low dose administration (Peter et al., 2000). Nevertheless, to make sure that this dose was not too low, we repeated the experiment in SOD1G93A mice with a dose of 10 mg/kg. Upon measuring the lifespan of this high-dose cohort of
animals, we consistently saw no difference in the MB-treated animals compared with saline-treated animals (MB: mean = 137.5, saline: mean = 141.0, P = 0.3268).

Lougheed and Turnbull (2011) did not report the physiological outcomes of the treatment. Hence, the possibility that MB could have ameliorated some aspects of the disease has not been addressed in that study. Here, we have examined the major pathological hallmarks of ALS in SOD1<sup>G93A</sup> and in TDP<sup>43<sup>G348C</sup> mice. First, the number of motor neurons remained unchanged in SOD1<sup>G93A</sup> (Fig. 4) after MB treatments. Besides, the extent of misfolding and aggregation of SOD1 proteins correlates with the disease in mutant SOD1 mice (Gurney, 1997; Bruijn et al., 1998; Wang et al., 2005; Prudencio et al., 2009). Accordingly, we investigated that feature and we showed that treatment with MB does not reduce aggregation/misfolding of SOD1 (Figs. 3A and 5).

There is evidence that misfolded SOD1 may be a common feature of most ALS patient, familial or even sporadic (Bosco et al., 2010; Forsberg et al., 2010). However, it has been proposed that sporadic ALS cases with TDP-43 abnormalities have a different etiology than familial ALS caused by SOD1 (Neumann et al., 2006; Orrell, 2010). Consequently, the possibility that MB may have protective effects in ALS of other etiologies than mutant SOD1 could not be excluded. For instance, MB was able to clear aggregates of TDP-43 in a cellular model of ALS (Yamashita et al., 2009). In contrast, we report that MB had no effect on disease symptoms or on formation of cytoplasmic TDP-43 inclusions in transgenic mice expressing TDP-43<sup>G348C</sup>.

The TDP-43<sup>G348C</sup> mice develop during aging motor dysfunction, a main feature of ALS disease (Swarup et al., 2011). The TDP-43<sup>G348C</sup> mice treated with MB for 6 months exhibit...
ited the same motor performance as saline-treated TDP-43G348C mice (Fig. 2). Moreover, immunodetection of cytoplasmic TDP-43, of ubiquitin and of inflammation remained unchanged by the MB treatment (Fig. 3B, D).

The fact that lithium did not alleviate ALS symptoms in SOD1G93A mice is concordant with recent negative results by independent groups (Gill et al., 2009; Pizzasegola et al., 2009; Aggarwal et al., 2010; Chio et al., 2010). These negative results were based on studies with larger cohorts of mice than earlier positive studies (Shin et al., 2007; Feng et al., 2008; Fornai et al., 2008). The scope of our experiments was mainly to investigate the possible synergic effect of MB with Li, because both were shown to achieve such effects in other paradigms (Shin et al., 2007; Feng et al., 2008; Bahremand et al., 2010). Based on our results we concluded that this was not the case in G93A mice and consequently we did not pursue further lithium tests in another ALS mouse model, the TDP-43 transgenic mice.

It is surprising that despite the existence of potential therapeutic targets for MB in ALS pathology, this compound could not improve the disease phenotypes in two different mouse models, the G93A mice and TDP-43 mice. There are still much uncertainties about pathogenic mechanisms that contribute directly to neurodegeneration process (Chattopadhyay and Valentine, 2009; Barber and Shaw, 2010; Baumer et al., 2010; Bogaert et al., 2010;
Lagier-Tourenne et al., 2010; Ticozzi et al., 2010; Pizzuti and Petrucci, 2011). Therefore, a possible explanation for the MB failure to alleviate ALS features could be that MB targets such as NOs, oxidative stress, mitochondria deficits, and protein aggregation are not the key contributors of motor neuron loss in these mouse models.

In summary, our results suggest that, despite its recognized protective properties (Schirmer et al., 2011), MB is inappropriate for treatment of ALS associated with either SOD1 mutations or TDP-43 abnormalities.

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