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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 efficient 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, SOD1^{G93A} mice and TDP-43^{G348C} transgenic mice. Intraperitoneal administration of MB at two different doses was initiated at the beginning of disease onset, at 90 days of age in SOD1^{G93A} and at 6 months of age in TDP-43^{G348C} mice. Despite its established neuroprotective properties, MB failed to confer protection in both mouse models of ALS. The lifespan of SOD1^{G93A} mice was not affected by MB treatment. The declines in motor function, reflex score, and body weight of SOD1^{G93A} 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 SOD1^{G93A} mice. In TDP-43^{G348C} mice, MB failed to improve motor function. Cytosolic translocation of TDP-43, ubiquitination and inflammation remained also unchanged after MB treatment of TDP-43^{G348C} mice.

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 undistinguishable. Twenty percent of the familial cases are related to mutations in the Cu/Zn superoxide dismutase gene (*SOD1*) gene, although the mechanisms leading to pathology remain unclear (Boillee 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 Bogaert 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 SOD1^{G93A} 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; Narsapur and Naylor, 1983; Eroglu 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; Pizzasegola et al., 2009) and humans (Aggarwal et al., 2010; Chio et al., 2010). Various hypoth-

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Abbreviations: ALS, amyotrophic lateral sclerosis; DRG, dorsal root ganglia; IR, immunoreactivity; MB, methylene blue; SOD1, Cu/Zn superoxide dismutase; VR, ventral root.

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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 SOD1^{G93A}, 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-43^{G348C} 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

SOD1^{G93A} mice [stock number 002726] were acquired from the Jackson Laboratory (Bar Harbor, ME, USA) and enriched in C57BL/6HSd strain ($n>20$). SOD1^{G93A} mice were genotyped in accordance with Jackson Laboratory protocols. SOD1^{G93A} mice were injected at the beginning of the symptomatic stage (90 days) every 2 days until their death. TDP-43^{G348C} mice were generated in C3B6 background (described in Swarup et al., 2011) and backcrossed in C57BL/6 background for at least 10 generations. TDP-43^{G348C} mice were injected at the beginning of the symptomatic stage (6 months) every 2 days until 12 months. Methylene blue (Sigma, St-Louis, MO, USA) was dissolved in 0.9% saline, and mice were given 1 mg/kg (SOD1^{G93A} and TDP-43^{G348C} mice) or 10 mg/kg (SOD1^{G93A} mice) intraperitoneally. Lithium (Sigma) was dissolved in 0.9% saline and injected intraperitoneally at a dose of 10 mg/kg (SOD1^{G93A} 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 SOD1^{G93A} 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 SOD1^{G93A} 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-43^{G348C} and SOD1^{G93A} 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 SOD1^{G93A} and TDP-43^{G348C} 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 SOD1^{G93A} 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-43^{G348C} 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-488 or Alexa-594 (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 tetraoxide 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 SOD1^{G93A} 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 protein was verified by stripping 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-