NEUROSCIENCE
INTERNATIONAL BRAIN IBRO RESEARCH ORGANIZATION

RESEARCH ARTICLE

K. C. Biju et al. / Neuroscience xxx (2018) xxx–xxx

Methylene Blue Ameliorates Olfactory Dysfunction and Motor Deficits in a Chronic MPTP/Probenecid Mouse Model of Parkinson's Disease

K. C. Biju,[a] Robert C. Evans,[a] Kripa Shrestha,[a] Daniel C. B. Carlisle,[a] Jonathan Gelfond[b] and Robert A. Clark[a,c]*

[a] Department of Medicine, University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, TX 78229, United States

[b] Department of Epidemiology and Biostatistics, University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, TX 78229, United States

[c] South Texas Veterans Health Care System, 7400 Merton Minter Blvd, San Antonio, TX 78229, United States

Abstract—Mitochondrial dysfunction and oxidative stress are very prominent and early features in Parkinson's disease (PD) and in animal models of PD. Thus, antioxidant therapy for PD has been proposed, but in clinical trials such strategies have met with very limited success. Methylene blue (MB), a small-molecule synthetic heterocyclic organic compound that acts as a renewable electron cycler in the mitochondrial electron transport chain, manifesting robust antioxidant and cell energetics-enhancing properties, has recently been shown to have significant beneficial effects in reducing nigrostriatal dopaminergic loss and motor impairment in acute toxin models of PD. However, no studies have investigated the impact of this promising agent in chronic models or for olfactory dysfunction, an early non-motor feature of PD. To test the efficacy of low-dose MB for olfactory dysfunction, motor symptoms, and dopaminergic neurodegeneration, mice were injected with ten subcutaneous doses of 25 mg/kg MPTP, plus 250 mg/kg intraperitoneal probenecid or saline/probenecid at 3.5-day intervals. Following the onset of olfactory dysfunction, MPTP/probenecid (MPTP/p) and saline/probenecid mice were provided drinking water with or without 1 mg/kg/day MB. Oral delivery of low-dose MB significantly ameliorated MPTP/p-induced deficits in motor coordination, as well as degeneration of tyrosine hydroxylase (TH)-positive neurons of the substantia nigra and TH-positive terminals in the striatum. Importantly, olfactory dysfunction was ameliorated by MB treatment, whereas this benefit is not observed with currently available anti-Parkinsonian medications. These results indicate that low-dose MB is a promising neuroprotective intervention for both motor and non-motor features of PD. Published by Elsevier Ltd on behalf of IBRO.

Key words: neurodegeneration, Parkinson's disease, olfactory dysfunction, dopamine, nesting behavior, substantia nigra.

INTRODUCTION

Parkinson's disease (PD) has long been viewed as a motor system disorder, promoting an era of therapeutics focused almost exclusively on dopamine neurons and motor symptoms. However, the disease is accompanied by numerous non-motor manifestations that add significantly to the overall level of disability (Marras and Chaudhuri, 2016). Current PD treatment, based primarily on pharmacological replacement of dopamine to treat motor symptoms, provides only symptomatic relief that typically wanes in efficacy after a few years. As therapeutic effectiveness diminishes, patients begin to suffer from drug-resistant motor symptoms (speech impairment, abnormal posture, gait and balance problems), as well as increasing drug side effects (psychosis, motor fluctuations, dyskinesia). Thus, replacing lost dopamine is clearly insufficient for arresting the disease progression. In fact, many non-motor symptoms (olfactory, sleep, and autonomic dysfunctions, anxiety, depression) precede the motor symptoms by years, or even decades (Pfeiffer, 2016; Schapira et al., 2017). Since non-motor symptoms are likely caused by the same protein aggregation pathologic mechanism that leads to motor symptoms, this temporal pattern begs the question of whether disease-modifying therapeutic strategies aimed at non-motor symptoms during the prodromal stage might also prevent or delay dopaminergic neurodegeneration and motor symptoms, thereby providing much-needed therapeutic benefit.

*Correspondence to: R. A. Clark, Department of Medicine, University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, TX 78229-3900, United States. Fax: +1-210-567-4654.
E-mail address: clarkra@uthscsa.edu (R. A. Clark).

Abbreviations: 6-OHDA, 6-hydroxydopamine; L-DOPA, L-3,4-dihydroxyphenylalanine; MB, methylene blue; MPP+, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; OD, optical density; p, probenecid; PD, Parkinson's disease; SNpc, substantia nigra pars compacta; SNpr, substantia nigra pars reticulata; STRdl, dorsolateral region of striatum; STRv, ventral region of striatum; TH, tyrosine hydroxylase.

https://doi.org/10.1016/j.neuroscience.2018.04.008
0306-4522/Published by Elsevier Ltd on behalf of IBRO.

Methylene blue (MB) (methylthioninium chloride) is a small-molecule synthetic heterocyclic organic compound. At low levels (nanomolar), MB acts as a renewable electron cycler in the electron transport chain, thereby enhancing cytochrome oxidase activity and ATP production, promoting cell survival (Rojas et al., 2012). MB also decreases production of reactive oxygen species in the electron transport chain via bypassing complex I/III activity. Hence, MB has the potential to mitigate oxidative damage. Indeed, mitochondrial impairment and oxidative stress are very prominent features in the PD brain (Lavrovsky et al., 2000; Ahlskog, 2005). Tissue samples from human PD patients provide convincing evidence for oxidative damage to a broad range of macromolecules, such as nucleic acids, lipids, and proteins (Sanders and Greenamyre, 2013). Moreover, *in vitro* and *in vivo* studies suggest that oxidative stress can induce α-synuclein aggregation (Hashimoto et al., 1999; Kowall et al., 2000), an early event in the initiation of PD. Interestingly, the olfactory bulb, one of the two brain regions where α -synucleinopathy first appears, is also very susceptible to mitochondrial compromise, oxidative stress, and excitotoxicity. Furthermore, olfactory dysfunction is an early warning sign, with olfactory loss occurring in up to 90% of PD patients. Although the precise mechanisms of mitochondrial dysfunction and oxidative stress in the etiology or pathogenesis of PD are yet to be elucidated, available data suggest that they contribute significantly to neurodegeneration in PD (Sanders and Greenamyre, 2013). In addition to acting as a renewable electron cycler in the mitochondrial electron transport chain, MB exerts its effect through other mechanisms relevant to PD – e.g., inducing autophagy, promoting neurogenesis, elevating monoamine levels through inhibition of monoamine oxidases, inhibiting nitric oxide synthase and nitric oxide-sensitive soluble guanylate cyclase, and blunting inflammatory responses (Deutsch et al., 1996; Sontag et al., 2012; Guerrero-Munoz et al., 2014).

Recently, MB has been shown to have significant beneficial effects in reducing nigrostriatal dopaminergic loss, motor impairment, and attentional deficits in acute toxin models of PD (Rojas et al., 2009; Wen et al., 2011; Smith et al., 2017). However, no studies have investigated the impact of this promising agent in chronic models or for olfactory dysfunction, an early non-motor feature of PD. Typically, non-motor features precede motor symptoms. To the extent that non-motor and motor deficits share similar mechanisms, drugs that mitigate non-motor symptoms (e.g., olfactory dysfunction) should have a better chance of being disease modifying than those that address only motor symptoms, provided the treatment is initiated at an early stage. Thus, olfactory efficacy may forecast the prevention or delay in onset of motor dysfunction in clinical trials of novel disease-modifying drugs. Additionally, to the best of our knowledge, this is the first study on the effect of MB on a chronic MPTP/probenecid mouse model of PD. Given the diverse pathological processes and heterogeneity in the expression and progression of the clinical manifestations of PD, as well as the likelihood that there are multiple contributing etiologic factors, there is no single ideal animal model. Therefore, testing of promising drug candidates in multiple models is critical for assessing predictive value of successful translation of drugs into the clinic.

EXPERIMENTAL PROCEDURES

Mice

Male C57BL/6J mice at 10 weeks of age were group-housed in a 14/10-h light/dark cycle. Animal husbandry was in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, Society for Neuroscience Policies on the Use of Animals and Humans in Neuroscience Research, and institutional requirements for animal care. A total of 30 mice were used. Baseline general activity in the open field, as well as proper righting reflex, were assessed before the experiment. All treatments and behavioral tests were performed during the light cycle. For the behavioral tests the mice were acclimatized to the testing room for at least 1 h prior to the session. The smallest possible number of mice was used (based on power calculations) and all efforts were made to minimize suffering.

MPTP/probenecid treatment

One week after the baseline assessment of general activity and righting reflex, the mice in one group were treated with ten subcutaneous doses of 25 mg/kg MPTP (Sigma, St. Louis, MO, USA) in saline, plus 250 mg/kg intraperitoneal probenecid (Sigma) in Tris–HCl buffer at 3.5-day intervals for a total of five weeks (MPTP/p group; $n = 20$; Fig. 1) (Meredith et al., 2008). Mice in the other group received saline and probenecid on the same schedule and were used as controls (Saline/p group; $n = 10$).

MB treatment

To assess the therapeutic effects of MB in early-stage disease, eleven days after the first dose of MPTP/p the animals were screened for olfactory dysfunction by recording the time spent in familiar versus unfamiliar compartments (Prediger et al., 2010). Following the onset of olfactory dysfunction (day 11 post MPTP/p), one subgroup from each of the MPTP/p ($n = 10$) and Saline/p ($n = 5$) mice were given 1 mg/kg/day MB, USP (Akorn, Lake Forest, IL, USA) in drinking water (Hosokawa et al., 2012), whereas the remaining MPTP/p ($n = 10$) and Saline/p ($n = 5$) mice were given regular drinking water (Fig. 1). Of the 20 mice in the MPTP/p arm 5 died of acute toxicity during MPTP/p treatment, thus reducing the total number in the MPTP/p arm to 15 (7 MPTP/p and 8 MPTP/p + MB, as shown in Fig. 2). Body weight and liquid consumption were monitored throughout the course of MB treatment. Water intake averaged between 4.3 and 4.7 ml/30 g body weight/day. MB did not induce any significant change in liquid consumption. The selected concentration of MB in the drinking water was based on the assumption that a 30-g mouse drinking 4.5 ml of liquid/day would receive MB at 1 mg/kg body

Please cite this article in press as: Biju KC et al. Methylene Blue Ameliorates Olfactory Dysfunction and Motor Deficits in a Chronic MPTP/Probenecid Mouse Model of Parkinson's Disease. Neuroscience (2018), https://doi.org/10.1016/j.neuroscience.2018.04.008