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The neuroprotective action of lenalidomide on rotenone model of Parkinson's Disease: Neurotrophic and supportive actions in the substantia nigra pars compacta

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ABSTRACT

Lenalidomide is a centrally active thalidomide analog that has potent anti-inflammatory and antiangiogenic activities. Currently, it is primarily used in the treatment of multiple myeloma and myelodysplastic syndromes. However, recent studies have revealed in addition to neuroprotection and neuromodulation of lenalidomide. Because of this combination of inflammation and neuro-immunogenic properties, lenalidomide is considered as a high potential compound for the treatment of neurodegenerative diseases. Despite intensive research during the last decade, the role of neurotrophic elements in the effect of lenalidomide is still not well understood. Therefore, in the current study, the effects of lenalidomide on neurodegeneration were investigated in a rotenone model of Parkinson's disease (PD) rat model. The PD rat model was generated by rotenone injection into the substantia nigra pars compacta (SNpc). After validation of the PD model, the rats were treated with lenalidomide (100 mg/kg) for 28 days. Our data shows that lenalidomide alleviated rotenone-induced motor impairments and deficits in dopamine-related behaviors and resulted in increased levels of tumor necrosis factor- α and calcium-binding protein B in the SNpc. Moreover, chronic lenalidomide treatment resulted increase in transforming growth factor immunoreactivity and brain derived neurotrophic factor expression in the SNpc. In addition, chronic treatment mitigated tyrosine hydroxylase expression prevented the rotenone-induced decrease in dopamine levels, and consequently a decrease in caspase-3/9 immunoreactivity. This thus shows that chronic lenalidomide treatment improves neuronal survival. Together with our data demonstrate that lenalidomide, in addition to its anti-inflammatory and immunomodulatory actions, is also capable of increasing neurotrophic factors in the SNpc, thereby preventing rotenone-induced motor impairments.

1. Introduction

Parkinson's disease (PD) is the most common neurodegenerative disorder after Alzheimer's disease. It is characterized by degeneration of the dopaminergic neurons and, depletion of dopamine levels in the substantia nigra pars compacta (SNpc). Due to the loss of dopaminergic neurons, tremor, rigidity, bradykinesia are key symptoms of PD. However, these symptoms first appear in a later stage of degeneration, which makes diagnosis difficult in the early stages [1]. Despite the available

treatment options, there is currently no known cure nor compounds that completely block neurodegeneration.

Several molecular mechanisms have been linked to the onset of neurodegeneration in PD, however, none of them can solely explain this complex disorder. An emerging hypothesis in PD implicates critical roles of neuroinflammation and the peripheral and central inflammatory response [2,3]. Chronic neuroinflammation and its elements are accepted as hallmarks of PD progression [4]. After the demonstration of increased microgliosis and astrogliosis in the post-mortem PD patients,

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new evidence indicates sustained neuroinflammatory products are detrimental to dopaminergic neuronal survival [5,6]. Cellular and molecular studies also demonstrated an increased inflammatory profile in PD animal models [7,8]. Furthermore, all the PD-inducing toxins 6-OHDA (6-hydroxydopamine), MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) and rotenone induce massive inflammatory responses, including increase in pro-inflammatory cytokines, upregulation of the expression of the cellular inflammatory elements and intensifying peripheral and central immune responses. Consistently, compounds that counteract the effect of these PD-inducing toxins showed improved motor performance and alleviated the loss of dopaminergic neurons in these experiments [9]. Dexamethasone, a synthetic potent steroid, showed neuroprotective effects in MPTP and lipopolysaccharide (LPS) treated mice [10]. Additionally, several non-steroidal drugs have been proposed inhibiting the degeneration of dopaminergic neurons in PD [11]. Aspirin is a potent inhibitor of cyclooxygenase-2 (COX-2), it also prevents dopaminergic neuronal loss and strengthens the resolution phase of inflammation in a 6-OHDA-induced PD rat model [12]. Altogether, compounds targeting the inflammatory response in the central nervous system show high potential for treating PD.

In addition to the neuroinflammation, neurotrophic support is vitally essential for dopaminergic neuronal survival [13,14]. Neurotrophic factors such as brain derived neurotrophic factor (BDNF), glial derived neurotrophic factor (GDNF), and nerve growth factor (NGF) are crucial for cell survival in the mesencephalic neurons. Importantly, PD patients have decreased levels of these neurotrophic factors [15]. Furthermore, exogenous administration of BDNF increases the number and survival of dopaminergic neurons, but clinically obstacles are not overcome yet [16]. Moreover, the levels of other trophic factors for neuronal plasticity, including bone morphogenic protein (BMP), transforming growth factor- β (TGF- β), transforming growth factor- α (TGF- α), fibroblast growth factor (FGF), ciliary neurotrophic factor (CNTF), are important for neuronal survival and are altered in PD patients [17,18].

Lenalidomide is a thalidomide analog that belongs to the group of immunomodulatory imide drugs (IMiD). It has high bioavailability and good blood-brain barrier permeability, and it has potent anti-inflammatory and anti-angiogenic effects [19]. Currently, Lenalidomide is primarily used for the treatment of multiple myeloma (MM) and myelodysplastic syndromes (MDS), however, several studies have reported that lenalidomide also has neuroprotective effects, including in experimental models for PD [20]. In this respect, lenalidomide-mediated neuroprotection and antiinflammatory action has been shown in BV-2 microglial cells and mThy1- α -syn induced transgenic animals with reducing effects on maladaptive neuroinflammation [21].

Therefore, compounds that are regulating the level of these trophic factors could be beneficial to the treatment of PD. To further explore these compounds, it is crucial to completely understand their effects and cellular mechanisms. Therefore, the aim of this study is to characterize the effects of lenalidomide in a rotenone model of PD model in respect of neuronal survival.

2. Material and methods

2.1. Animals

The animals used in this study were obtained from the Ondokuz Mayıs University Vivarium after approved by the Animals Ethics Committee of Ondokuz Mayıs University (HADYEK 2020-07). Thirty male Wistar Albino rats (220 ± 20 g) were maintained under proper conditions ($22 \pm 2^\circ\text{C}$, $55 \pm 3\%$ humidity, 12/12 day and night cycle), and all efforts were made to reduce animal suffering. All experiments were done according to the Declaration of Helsinki for Experimental Animals.

2.2. Chemicals

Rotenone, lenalidomide, apomorphine hydrochloride, dimethyl

sulfoxide (DMSO), and methylcellulose were obtained from Sigma-Aldrich Inc. (St. Louis, MO). Lenalidomide was dissolved in 0.5% methylcellulose and administered (100 mg/kg, p.o) in a 5 mL/kg volume. Apomorphine and rotenone were freshly dissolved in phosphate-buffered saline (PBS, pH 7.4) and DMSO, respectively.

2.3. Rotenone injection and experimental groups

Animals were anesthetized with ketamine and xylazine hydrochloride (80 mg/kg-4 mg/kg) and placed in a stereotaxic apparatus (World Precision Instruments, USA). After shaving the head of the animals, short incisions were made through the frontal-occipital direction. According to the rat brain atlas, after determining bregma, one burr hole was drilled on the following coordinates; SNpc AP:5.0 mm, M-L:2.00 mm, DV:8.0 mm [22]. Rotenone (5 $\mu\text{g}/\text{rat}$) was injected through the burr hole with a 28 G Hamilton syringe. For complete drug diffusion, the syringe was removed 2 min after injection for complete diffusion. Animals in the control group were injected with DMSO (1 μL) as a rotenone vehicle. Next, the hole was filled with bone cement and antiseptis was performed. Animals were observed for at least three days for abnormal behaviors. Ten days after the rotenone injection, created PD model was evaluated with the apomorphine-induced rotation test [23].

Generation of the model was considered successful when the animals showed the characteristic apomorphine-induced turning behavior. After apomorphine (1 mg/kg, s.c) administration animals were recorded by video recorder for 60 min, then analyzed 30 min period with 10 min interval and only the animals that turned more than 7 times were accepted as PD. Subsequently, the animals were divided into two equal groups, designated as lenalidomide and rotenone. The apomorphine test was repeated after every week for surveillance of dopamine-related neuronal damage (Supplementary material Fig. 1). Lenalidomide (100 mg/kg, p.o) was administered during a 28-day period, and the rotenone group was administered with 0.5% carboxymethyl cellulose (CMC) (5 mL/kg) as a lenalidomide vehicle. After 28-day treatment period animals of both groups were tested for behavioral parameters. The Lenalidomide dose was selected according to the previous reports in central nervous system experimental models [21,24,25].

2.4. Open field test

24 h after the last drug administration, all animals were placed individually on a plexiglass open field apparatus (72×72 cm area with 36 cm walls) for evaluation of the locomotor activity [26]. The floor of the apparatus was divided by lines into 16 squares (18×18 cm) plus one central square (18×18 cm). Locomotor activity of animals was evaluated by recording ambulation (the number of the squares that the animals crossed) and the immobility time (5 min).

2.5. Rotarod and cylinder test

Motor coordination and balance skills were evaluated with the rotarod test, while forelimb akinesia was analyzed by the cylinder test [27]. Before the tests, all animals had a two days pre-training to allow stable conditions on the rotarod apparatus [26]. For the rotarod test all animals were placed on a rotating rod apparatus in the opposite direction to the platform. During the 5-minute test period, the speed of the rotating rod was increased every minute by ten rpm and the length of time for which the animal remains on the rod (fall latency) was recorded. For the cylinder test the rats were placed in a plexiglass cylinder (30×20 cm), and the number of the forelimb contacts made by each limb to the apparatus was recorded for 5 min. Then the cylinder test score was calculated using the following formula; $[(\text{contralateral side} + 1/2 \text{ both}) / (\text{ipsilateral side} + \text{contralateral side} + \text{both})]$. A test score less than 0.5 indicates forelimb motor impairment.

2.6. Biochemical analysis

Following behavioral experiments, animals were euthanized first with high dose of ketamine/xylazine, transcardially perfused with heparinized-PBS and then decapitated. Brain tissue was extracted, and the SNpc area was isolated. Samples were homogenized with PBS (pH 7.4) in a glass homogenizer, and the protein contents were determined [28]. Homogenates were stored at -80°C until further analysis. The tumor necrosis factor- α (TNF- α), calcium-binding protein B (S100 β), and total dopamine levels were determined using commercially available ELISA kits (TNF- α assay kit #201-11-0765, S100 β assay kit #201-11-1151, Dopamine assay kit #201-11-0220 Sunred).

2.7. Western blot analysis

After SNpc area isolation, samples were homogenized with ice-cold RIPA (radioimmunoprecipitation assay) buffer in a glass homogenizer. The protein contents were determined with Lowry's method; equal amounts of proteins among different samples were separated by 4–20% SDS-PAGE [28]. Proteins were then transferred to PVDF (polyvinylidene difluoride) membranes and blocked in 5% bovine serum albumin (BSA) solution for preventing non-specific binding. Next, membranes were washed in TBS-T solution and incubated with primary antibodies (BDNF Abcam # ab226843, β -actin Cell Signaling #8457, Tyrosine hydroxylase (TH) Elabscience #E-AB-70077) at $+4^{\circ}\text{C}$ for overnight. Following three TBS-T (tris-buffered saline-tween20) washing steps, the membranes were incubated with secondary antibody (Bio-rad, Rabbit anti-Goat IgG (H + L)-HRP Conjugate #1721034) for an hour. Protein were visualized using enhanced chemiluminescence (ECL) substrate. Band intensities were quantified with ImageJ (NIH, US), and β -actin was used as internal standard.

2.8. Histopathology and immunohistochemistry analyses

During the necropsy, brain samples were harvested and fixed in 10% neutral formalin solution. After 2 days of fixation, brain samples were routinely processed by automatic tissue processor (Leica ASP300S, Wetzlar, Germany). The tissues were embedded in paraffin, and five- μm -thick cross-sections were taken from the paraffin blocks. The tissue sections were stained with hematoxylin-eosin (HE) and examined microscopically [29].

Selected sections were stained with antibodies against caspase-3 [Anti-caspase-3 antibody, (ab4051; Abcam-Cambridge, UK)]; caspase-9 [Anti-caspase-9 antibody (ab52298; Abcam-Cambridge, UK)], and transforming growth factor [TGF- α (D-6) antibody, (sc-374433; Santa Cruz Texas, USA)]. For visualization, a streptavidin-biotin peroxidase based, Ultravision Detection System Anti-Polyvalent, HRP/DAB (Ready-To-Use) (TP-015-HD) was used according to the manufacturer's instructions, and 3,3'-diaminobenzidine was used as the chromogen (Thermo scientific, Cheshire, UK). In this study, the primary antibodies were used at a 1:100 dilution. Primary antibody was omitted in negative controls. All IHC experiments were performed by a specialized pathologist blinded to the sample treatments. All brain samples, SNpc sections, were analyzed semiquantitatively as follows. Five different sections were examined in each sample, which then were scored from 0 to 3, according to the intensity (0, absence of staining; 1, slight; 2, medium, and 3, marked) [30]. After the routine microscopic examination, computer-assisted histomorphometric measurements and immunohistochemical scoring were obtained using an automated image analysis system (Olympus CX41, Olympus Corporation, Tokyo, Japan). The Database Manual CellSens Life Science Imaging Software System (Olympus Corporation) was used for evaluation of the lesioned area.

2.9. Statistical analysis

All experimental data were analyzed with SPSS (v21.0, Illinois, US). The normality of the data was determined with the Shapiro-Wilk's normality test and subsequently analyzed by Kruskal Wallis and one-way analysis of variance (ANOVA) tests. Tukey's test was performed for posthoc analysis. P values less than 0.05 are considered to be statistically significant.

3. Results and discussion

3.1. Lenalidomide reduces rotenone-induced motor impairment and dopamine-related behavioral deficits

In the recent decade it became clear that neuroinflammation plays an important role in the progression of PD. Therefore, many academics and

Fig. 1. Open field test, rotenone and cylinder tests results demonstrated lenalidomide alleviated motor impairment. Open field test results demonstrated lenalidomide increased ambulation (A), and decreased immobility time (B). Also, lenalidomide extended rotarod test falling latency (C) and ameliorated unbalanced use of contralateral forelimb in the cylinder test (D). All data are expressed as mean $\pm$ SD. *** $p < 0.001$ versus control group, ### $p < 0.001$, versus rotenone group. Results of all groups. SD: Standard deviation.

industry have focused on screening drugs with anti-inflammatory properties that can interfere with the constant neuroinflammatory response in PD [31]. Previous studies have shown that thalidomide and its derivatives, including lenalidomide, reduce neuroinflammation in PD. Here we further characterized the effects of lenalidomide on

neurodegeneration in a rotenone model of PD. Rotenone is a good model for PD research, since it is a potent complex I inhibitor that causes an imbalance of cellular respiration and inflammatory response, which results in neuronal death in the SNpc and motor neurons, similar to what is observed in a subset of PD patients [32,33]. For our study we used a group

Fig. 2. Representative histopathological figures of SNpc from the groups under H&E staining (A). Normal histological appearance in the control, decreased neuron number and degenerative neuron (thick arrow) and vacuolization (thin arrows) in the rotenone group, and amelioration of these pathological findings in the lenalidomide group were represented. Additionally, caspase-3 (B), caspase-9 (C) and TGF- α (D) immunoreactivity in SNpc between the groups were evaluated (B–D). Lenalidomide exerted neurotrophic and decreased cell death because of rotenone insult. Histopathological scores are expressed as mean $\pm$ SD. *** $p < 0.001$, ### $p < 0.001$ versus rotenone group. Negative expression in the control, marked increase in the caspase 3-9 (B, C) and TGF- α (D) expression in the rotenone and inhibited caspase-3-9 (B, C) expression increase and TGF- α (D) expression decrease in the lenalidomide, Bar = 50 μ m. TGF- α : Transforming growth factor

Fig. 3. ELISA and western blot results of the total dopamine (A), and TNF- α (B), S100 β (C) levels and BDNF (D), TH (E) expression in the SNpc of all groups. Decreased dopamine levels and BDNF and TH expression seen in the rotenone were diminished with lenalidomide treatment. Further, increased TNF- α (B) and S100 β (C) levels after rotenone insult were attenuated with lenalidomide. Blot pictures stand for at least 3 samples. All data are expressed as mean $\pm$ SD. *** p < 0.001 versus control group, ### p < 0.001, versus rotenone group.

TNF- α : Tumor necrosis factor- α ; BDNF: Brain derived neurotrophic factor; TH: Tyrosine hydroxylase; S100 β : Calcium-binding protein β .

of male Wistar Albino rat that were injected with rotenone, and another group with DMSO as control. Successful generation of the PD-model and the associated characteristic rotenone-mediated turning behavior was confirmed by an apomorphine-induced rotation test [23]. First, we investigated the effect of lenalidomide on rotenone-induced motor impairment and behavioral parameters [20,32]. The locomotor activity

of the animals was evaluated in an open field test by recording ambulation (the number of the squares that the animals had passed) and the immobility time. As illustrated in Fig. 1, rotenone injection induced a significant decrease in ambulation (Fig. 1A, p < 0.001) and increase in immobility time (p < 0.001, Fig. 1B). Interestingly, the animals that were treated with Lenalidomide (100 mg/kg) during a 28-day period, showed

a significant reduction in the effect of rotenone on ambulation (Fig. 1A, $p < 0.001$), as well as on the immobility time (Fig. 1B, $p < 0.001$). Next, the motor coordination and balance skills were measured with the rotarod test. Rotenone-induced significant motor impairment and a decreased falling latency, the length of time for which the animal remains on the rod (Fig. 1C). The cylinder test results show that rotenone caused significant ($p < 0.001$) alteration in forelimb touches, which is directly related to dopaminergic neuronal imbalance (Fig. 1D). Importantly, lenalidomide treatment significantly reduced the rotenone-induced motor impairment (Fig. 1C, $p < 0.001$) and improved the aggravated rear defects (Fig. 1D, $p < 0.001$). Together these results thus show that Lenalidomide has a protective effect on rotenone-induced locomotor defects, motor impairment and dopamine-related behavioral deficits.

3.2. Lenalidomide reduces rotenone-mediated neuronal death and neurogenesis

To directly assess the neuroprotective effect of lenalidomide, we performed histopathological examination. While normal SNpc histology was observed in the vehicle-treated animals, decreased neuron numbers, numerous of degenerated neurons and vacuolations were seen in the rotenone injected group (Fig. 2A). These rotenone-induced pathological defects became less severe after lenalidomide treatment (Fig. 2A-C). To further quantify neuronal cell death, we analyzed caspase-3/caspase-9 expressions, the major pathway responsible for dopaminergic neuronal loss in the SNpc [33]. Consistent with the observed neuronal death in the SNpc, our data reveal that rotenone significantly ($p < 0.001$) increased caspase-3/caspase-9 activation (Fig. 2B, C). Additionally, a significant ($p < 0.001$) decrease in TGF- α , which is a critical element for neuronal integrity [14], immunoreactivity, and immunohistochemical score in the SNpc were observed in the rotenone injected animals. Lenalidomide treatment significantly ($p < 0.001$) inhibited the rotenone mediated activation of caspase-3 and caspase-9, as well as improved TGF- α immunoreactivity (Fig. 2B, C, D).

3.3. Lenalidomide inhibited the rotenone-induced alterations on the total dopamine and TNF- α , S100 β levels

To further understand the neuroprotective molecular mechanism of lenalidomide on rotenone insults, we measured the level of dopamine, TNF- α and S100 β . TNF- α is a prominent cytokine and a critical component of the neuroinflammatory response in PD pathology [34]. Furthermore, accumulating data support a direct role of TNF- α in neuroplasticity and neuro-immune communication during PD progression [35]. S100 β is a well-known calcium-binding protein of which increased levels have been detected in post-mortem tissues of patients with neurodegenerative disorders, especially with PD [36]. Increased levels of S100 β can cause neuronal apoptosis, presumably through a mechanism involving nuclear factor- κ B [37]. On the other hand, Sathe et al. demonstrated that the depletion of S100 β works neuroprotective in MPTP induced PD model [38]. Our data shows a significant decrease in total dopamine levels (Fig. 3A, $p < 0.001$) in the rotenone group, while TNF- α (Fig. 3B, $p < 0.001$) and S100 β (Fig. 3C, $p < 0.001$) are increased, compared to the control group. These data indicate that rotenone as expected induces inflammatory reactions and astrogliosis. Importantly, our data shows that lenalidomide treatment has a protective effect on this response (Fig. 3). Lenalidomide significantly ($p < 0.001$) attenuated the decrease in dopamine and it decreased the TNF- α levels, which may suggest a role in regulating neuronal loss and increased trophic factors, concordant with other studies. Furthermore, our data shows that Lenalidomide decreased S100 β levels, which might explain the lenalidomide mediated reduction in caspase-dependent neuronal loss and decreased levels of TH expression in the SNpc (Fig. 3E).

3.4. Lenalidomide rescues BDNF and TH expression in the SNpc

BDNF is a well-documented neurotrophic factor and its role has been extensively investigated in PD [39]. BDNF is a critical survival factor for midbrain dopaminergic neurons. BDNF also supports the survival of several immature interneurons, which suggests a role in dopaminergic signaling [40]. Post-mortem studies have demonstrated that decreased BDNF mRNA levels in the SNpc, and decreased levels of BDNF correlates with the severity of the disease related symptoms [41]. Because of this critical role of BDNF in the nigrostriatal neuronal survival and integrity during PD, we next studied the effect of lenalidomide treatment on BDNF expression in the SNpc (Fig. 3). Rotenone significantly reduced BDNF expression as shown by quantification of the Western blots (Fig. 3D, ($p < 0.001$)). Additionally, in line with the dopamine-related behavior test results and total dopamine levels, rotenone significantly ($p < 0.001$) decreased TH expression (Fig. 3E). This reduction of total dopamine levels and TH expression could be correlated with the inflammatory response and loss of trophic support in the SNpc. Importantly, lenalidomide treatment significantly ($p < 0.001$) reversed this rotenone mediated decrease in BDNF and TH expression, thereby further support its neuroprotective effects against the toxicity exerted by the rotenone.

4. Conclusion

Here we studied the neuroprotective effects of lenalidomide on neuronal survival and plasticity elements in rotenone model of PD. Our data revealed that lenalidomide decreased rotenone induced motor impairment and dopamine-related behavioral deficits. Subsequently we showed that lenalidomide attenuated the decrease of TH expression and increase of caspase-dependent apoptosis in the rotenone treated the SNpc. Our biochemical analysis revealed that lenalidomide reduced the loss of neurotrophic factor triggered by rotenone. Considering all the results, the present study shows that lenalidomide, in addition to its anti-inflammatory and immunomodulatory actions, is also capable of increasing neurotrophic factors in the SNpc, thereby reducing rotenone induced neuronal cell death and motor impairment.

Caspase inhibitors also reduce rotenone-mediated defects in cellular and *in vivo* PD models, however since apoptosis is a vital mechanism for all cells and tissues, it is dangerous to use these compounds as a treatment option [37,38]. Therefore, drugs that affect these elements with tissue-specific context might be a more viable strategy for the treatment. The neurotrophic properties of lenalidomide thus make it a potential candidate for future PD therapeutics.

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CRediT authorship contribution statement

Fatma Nihan Cankara: Conceptualization, Methodology, Writing - original draft. **Caner Günaydın:** Methodology, Writing - original draft. **Süleyman Sırrı Bilge:** Supervision, Writing - review & editing. **Özlem Özmen:** Investigation, Writing - review & editing. **Arjan Kortholt:** Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare no conflict of interest present in this study.

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