Chapter 1

Introduction

Parkinson's disease (PD), one of the widely reported neurodegenerative movement disorders is characterized by the loss of neurons in nigrostriatal dopaminergic pathway. The pathway consists of substantia nigra pars compacta (SNc) and striatum. Dopaminergic neuronal cell bodies are located in the SNc and nerve terminals with axons project to the striatum (Bernheimer et al., 1973; Dauer and Przedborski, 2003). Dopamine (DA) participates in motivation, learning and is directly involved in encoding movement. Motor symptoms, including postural instability, akinesia, gait, rigidity, bradykinesia and resting tremor along with non-motor symptoms, such as cognitive, sleep, sensorimotor and cardiac dysfunction are observed in patients (Jeter et al., 2018; Kalia and Lang, 2015; Lang and Espay, 2018; Park and Stacy, 2009). PD affects approximately 0.4-1% of people around 60-79 years of age and 1.9 % of people more than 80 years of age worldwide (Pringsheim et al., 2014). More than ten million people are living with PD, which is expected to double by 2040 (Kowal et al., 2013; Kwok et al., 2017). However, its etiology is largely unknown. DA deficiency is one of the various factors leading to PD symptoms (Lang and Espay, 2018). Tyrosine hydroxylase (TH) acts as rate-limiting enzyme in biosynthesis of DA (Haavik and Toska, 1998; Zhu et al., 2012). Dopamine transporters (DAT) are involved in the uptake of synaptic DA in order to maintain steady DA levels in synapse and regulate its presynaptic function (Gainetdinov et al., 1998; Sossi et al., 2007). During early PD cases, 30-50% decline in DAT levels is observed in patients and it reaches up to 50-70% as the disease progresses (Nutt et al., 2004; Sossi et al., 2007).

Insufficient knowledge of pathogenesis may be an important reason behind the unmet therapeutic need for PD. Pathogeneis of PD involves multiple neurotoxic

pathways. Toxins, genes, immune and environmental factors, α-synuclein pathology, β-glucocerebrosidase (GCase) enzymatic deficiency, oxidative stress and mitochondrial dysfunction are some of the factors involved (Gegg et al., 2012; Moore et al., 2005; Olanow et al., 2009). Mitochondrial dysfunction is associated with increment in the levels of mitochondrial and intracellular reactive oxygen species (ROS) which give rise to oxidative stress, one of the factors responsible for PD pathogenesis. Oxidative stress occurs due to imbalance between ROS and cellular antioxidant defense system and leads to high amount of oxidized lipids, proteins, deoxyribo nucleic acid (DNA) and ribo nucleic acid (RNA) in PD patients (Nakabeppu et al., 2007). Impairment in mitochondrial complex enzyme system and electron transport chain (ETC) is followed by the release of cytochrome-C from the mitochondria which further increase activities of proteins like caspase-9 and caspase-3 causing cell death (Elmore, 2007; Moore et al., 2005). Some neurotoxins like 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA) are also used to induce experimental PD in animals (Moore et al., 2005). Unilateral intrastriatal injection of 6-OHDA in rats is an established and well-validated experimental model of PD (Kirik et al., 1998). PD is incurable to date as all the available drugs provide only symptomatic relief (Fox et al., 2018). Hence, there is a need for novel PD targets along with suitable experimental models to investigate new therapeutic compounds for PD.

Levodopa (L-DOPA; L-3,4-dihydroyphenylalanine) has been the gold-standard therapy for symptomatic treatment of PD and usually well tolerated. However, after long term use with high doses, it shows several side-effects like nausea, vomiting, motor fluctuations, dyskenesia and non-motor symptoms (Smith et

al., 2012). Various drugs are being investigated for their neuroprotective effects in PD (Müller, 2012). Monoamine oxidase B (MAO B) inhibitors, DA agonists, N-methyl-D-aspartate (NMDA) antagonist, anticholinergics, catechol-O-methyltransferase (COMT) inhibitors and dopamine decarboxylase inhibitors have been marketed for PD management. However, these drugs also cause various side effects like raised blood pressure, hepatotoxicity, oedema, orthostatic syndrome, inflammatory reactions, insomnia, depression and motor complications such as wearing off phenomena and dyskinesia. Therefore, various treatment approaches have been developed in order to circumvent the shortcomings of available drug therapies (Burn, 2018). The search for safe and effective drugs is crucial for management of PD. Several clinical trials for novel neuroprotective agents and supplementary drugs are ongoing for the same (Athauda et al., 2017; Lhommée et al., 2018; Reglodi et al., 2017).

β-Glucocerebrosidase (GCase) encoding gene GBA1 mutation numerically constitutes the major risk factor for PD (Bendikov-Bar et al., 2013; Schapira, 2015) which is more prevalent in early than late-onset PD cases (Sidransky et al., 2009). The activity of GCase, a lysosomal enzyme is not only decreased in PD patients but also in healthy subjects gradually with age (30-50 %). It becomes comparable to PD patients by about 70 years of life, due to which older people are susceptible to PD. There is a continuous decrease in lysosomal function with normal aging causing accumulation of misfolded proteins and dysfunctional mitochondria with α-synuclein pathology which turns out pathological by the seventh decade of life. Therefore, reduced GCase activity stimulates PD symptoms in non-GBA carriers (Rocha et al., 2015a). This indicates the need of GCase-targeting drugs for the management of PD.

Augmenting CNS (central nervous system) GCase activity has been evolved as therapeutic PD strategy in the recent years (Sardi et al., 2013). GCase – lysosome – α-synuclein pathways are being targeted to treat PD (Schapira, 2015) and novel GCase-activating drugs are entering clinical trials (Silveira et al., 2019). Different GCase activators either alone or in combination with other anti-PD drugs are being studied for the treatment of PD (Sardi et al., 2018). The discovery of the link between GBA mutations and PD has introduced a novel area for therapeutic investigation, with the possibility of targeting GCase as an important contributor to α-synuclein accumulation and mitochondrial dysfunction in the disease.

Ambroxol, an FDA (Food and drug administration)-approved drug is used against respiratory diseases (Stojkovska et al., 2018) as mucolytic therapy in productive cough or viscous mucus in bronchopulmonary conditions (Kakkar et al., 2018). Ambroxol is currently in clinical trial to stimulate GCase activity in phase-II study for PD [ClinicalTrials.gov Identifier NCT02914366 (Velayudhan et al., 2017) and NCT02941822] (Girolamo et al., 2017). There is unavailability of reports indicating the ambroxol-induced modulation of PD symptoms using *in vivo* PD models. This may be due to the lack of non-genetic PD models to mimic GCase deficiency. Considering the need of disease-modifying treatment in PD, repurposing the drugs is of utmost importance. Rebamipide, a gastrointestinal protective drug is reported to decrease Aβ43-lowered cell viability *in vitro* (Fukui et al., 2017). Additionally, it is reported to provide mitochondrial protection in various disease models *in vivo* and *in vitro,* including hepatic ischemia/ reperfusion injury (Diao et al., 2012; Gendy et al., 2017; Ishihara et al., 2010; Nagano et al., 2005). The drug crosses blood brain barrier and acts as anti-oxidant (Fukui et al., 2017; Kim and

Hong, 1995; Moon et al., 2012; Shioya et al., 1989). Rebamipide is reported to increase nuclear Nrf2 (nuclear factor erythroid 2-related factor 2) activity in collagen-induced arthritis (Moon et al., 2014) and in reflux esophagitis model *in vivo* (Song et al., 2016). Activation of nuclear Nrf2 activity is required to act against oxidative damage and mitochondrial dysfunction observed in PD (Joshi and A Johnson, 2012). Therefore, rebamipide may also act against oxidative stress, mitochondrial dysfunction and related GCase deficiency like factors involved in PD pathogenesis through Nrf2-mediated pathway.

Disease-modifying medications are unmet medical requirement in the treatment of neurodegenerative disorders (Francardo et al., 2017; Reidling et al., 2018). Due to unavailability of medical intervention to alter the disease-course by slowing and arresting its progression, the progressive death of dopaminergic neurons in SNc leads to two to three times increased mortality within 20 years from the onset of disease compared to general community (Dauer and Przedborski, 2003; Lang and Espay, 2018). Disease-modification has been attempted to achieve in PD by various drugs, such as AT2101 (Richter et al., 2014; Wrasidlo et al., 2016), while others are in clinical trials like PX002/RG7935 (Jankovic et al., 2018) and nilotinib (Simuni et al., 2018). Ambroxol hydrochloride, a GCase chaperone is currently undergoing clinical trials for its disease-modifying property in PD as stated earlier (Silveira et al., 2019). GCase deficiency, mitochondrial dysfunction and α-synuclein pathology are inter-related to each other and responsible for cell death (Osellame et al., 2013); (Cleeter et al., 2013; Mazzulli et al., 2011). Ambroxol due to its GCase-stimulating activity (Bendikov-Bar et al. 2011) and rebamipide due to its action against mitochondrial dysfunction (Diao et al., 2012) may target multiple pathological events

leading to dopaminergic cell death, and therefore may show disease-modifying potential.

Rebamipide is highly lipophilic and hydrophobic drug (Ngo et al., 2017; Pradhan et al., 2015), due to which it may not be well absorbed over gastrointestinal membrane through passive diffusion (Ashford, 2017; Huang et al., 2008), but is able to permeate the blood brain barrier (Fukui et al., 2017). This is the reason that the drug has low oral bioavailability (4.8 ± 1.4%) (Shin et al., 2004) and required higher dose (30 and 100 mg/kg) to show gastric mucosal protection against ethanol-induced gastric mucosal damage and ischemia reperfusion in rats (Choi et al., 2013). Considering the progressive neurodegeneration in PD, reducing the dose and frequency of rebamipide is of utmost importance to act against PD model in rats. Among the newer methodologies for administration of drugs is the use of transdermal approaches. Various drugs are being formulated as transdermal patches in order to increase their bioavailability, such as class II drugs [duloxetine (Singh and Bali, 2016), mefanamic acid (Suksaeree et al., 2017), flurbiprofen (Idrees et al., 2014), diclofenac (Bhaskar and Pranav Kapoor, 2010), repaglinide (Prajapati et al., 2011), topiramate (Cherukuri et al., 2017), losartan (Baviskar et al., 2012), propranolol (Cilurzo et al., 2014)], class-I drugs [diltiazem (Parhi and Suresh, 2016), fluoxetine (Parikh and Ghosh, 2005), ketotifen (Chiang et al., 1998), salbutamol (El-Gendy et al., 2008), isosorbide (Zhao et al., 2007)], class II drugs [lamivudine (Ramadan et al., 2018), aliskiren (Sethi and Mazumder)] and class I/III drugs [isoniazid (Ahn et al., 2017)].

Transdermal drug delivery has advantages over other routes because transdermal patches deliver drugs directly into the circulatory system, bypassing the

gastrointestinal system. Drug is loaded inside a patch in a relatively high dosage and the patch is directly applied on the skin for the relatively longer period. Skin consists of epidermis (100-150 µm thick) as outer layer with no blood flow. Epidermis includes a layer within, namely stratum corneum, which plays important role in transdermal drug delivery. Dermis is present beneath the epidermis and contains group of capillaries for blood transportation throughout the body. Therefore, drugs having ability to penetrate stratum corneum, may reach blood stream directly through passive diffusion process (Sheth and Mistry, 2011). Epidermis is metabolically inert, the bioavailable factor is 1, indicating that the drug is absorbed completely (Chandrashekar and Rani, 2008). The diffusion of drug will be continued into the blood for longer duration due to higher concentration of drug in the patch compared to blood, maintaining constant drug concentration in blood flow (Sheth and Mistry, 2011). Rebamipide shows characteristics for transdermal delivery, such as partition coefficient (Chandrashekar and Rani, 2008; Obae et al., 2017), molecular weight (Shabbir et al., 2014), lipophilicity (Prausnitz and Langer, 2008), low oral bioavailability (Chandrashekar and Rani, 2008; Shin et al., 2004) and hydrophobicity (Naik et al., 2000). Therefore, it may be an ideal candidate for transdermal administration to obtain the desired pharmacological effects with low doses.

Therefore, the current study introduces the validation of 6-OHDA-induced model of PD to evaluate GCase-targeting compounds for management of PD. Ambroxol is observed for its action against 6-OHDA-induced temporal changes in GCase enzymatic activity and PD symptoms. Rebamipide is evaluated for its effects on GCase activity and 6-OHDA-induced toxicity in hemiparkinson's model of rats via Nrf2-mediated mechanism. Ambroxol and rebamipide are observed for their

possible neurorestorative potential. Additionally, transdermal patches of rebamipide are formulated, characterized and evaluated against 6-OHDA-mediated toxicity in rats. A battery of behavioral tests was performed to evaluate the motor symptoms, which include apomorphine-induced rotation, open field, rotarod, grip strength and bar catalepsy tests. Neurochemical measure of PD was done by estimating striatal DA and their metabolites. Mitochondrial function was assessed by MTT [3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide] reduction, mitochondrial complex enzymes activities and mitochondrial bioenergetics. As a function of oxidative stress, mitochondrial lipid peroxidation was estimated. GCase activity, α-synuclein concentration, TH and DAT levels were measured to evaluate PD pathology. Specific mechanistic studies were conducted by using pharmacological inhibitor of Nrf2 (Nrf2i; trigonelline) to inhibit its translocation into the nucleus (Arlt et al., 2013). Nuclear Nrf2 activity was estimated to observe the mechanism responsible for rebamipide action. Antioxidant system regulated by Nrf2 was evaluated by estimating reduced glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT). The concentration of rebamipide was estimated in plasma and cerebrospinal fluid (CSF) for drug-loaded transdermal patches. Nissl's staining was performed to quantify the loss of nigral cells. Intrinsic pathway of apoptosis was expressed by cytochrome-C, and caspase-9 proteins. Caspase-3 was expressed to show apoptosis.