Chapter 3

Effects of ambroxol and rebamipide on GCase activity *in vitro*

3.1. Introduction

GCase, a lysosomal hydrolase is synthesized in the endoplasmic reticulum (ER)-bound polyribosomes, translocated to ER for quality control process and folding state confirmation to undergo N-linked glycosylation. GCase is then directed towards Golgi apparatus and trafficked to lysosome by LIMP-2 (lysosomal integral membrane protein type-2) receptor (Bendikov-Bar et al., 2013; Bendikov-Bar et al., 2011; Erickson et al., 1985; Reczek et al., 2007). Animals with GBA1 gene mutation also showed symptoms of neurodegeneration (Mazzulli et al., 2011). Most of the PD cases are sporadic, whereas genetic form of the disease is only found in 5-10% of total PD patients (Lesage and Brice, 2009). Therefore GBA1 mutation may only present in 5-10% of PD patients, but decreased GCase enzymatic activity is found in sporadic patients also, making GCase a serious risk factor for PD (Gegg et al., 2012).

Different small molecular pharmacological chaperones that are competitive GCase inhibitors bind to misfolded proteins in ER. Ambroxol, a pH-dependent and mixed-type GCase inhibitor acts as a chaperone to convert GCase to its full-length form and facilitates its trafficking through the ER. It bind to misfolded GCase in ER, take part in their folding process and enable them to bypass the ERQC [endoplasmic reticulum quality control] and ERAD [ER associated degradation] pathways followed by the trafficking of GCase-chaperone complex to lysosome (Bendikov-Bar et al., 2011; Maegawa et al., 2009; Schapira, 2015), where this complex gets dissociated because of elevated substrate concentration and low pH (Pereira et al., 2018), thereby restoring GCase activity in the lysosome. It binds best at the neutral pH of the ER where it helps with folding and trafficking, and poorest at the acidic pH of the lysosome, where it is no longer needed. This is the reason that the maximum

inhibition of GCase by ambroxol was reported at the pH of ER whereas GCase was not inhibited at lysosomal pH (Bendikov-Bar et al., 2011; Maegawa et al., 2009). Mitochondrial dysfunction and GCase deficiency share bidirectional relationship by increasing each-other, which leads to cell death (Cleeter et al., 2013; Mazzulli et al., 2011; Osellame et al., 2013). Rebamipide, a gastrointestinal protective drug due to its action against mitochondrial dysfunction (Diao et al., 2012) may target pathological events leading to dopaminergic cell death as discussed in **Figure 2.1** (page 13). However, there are no reports showing the effects of rebamipide on GCase enzymatic activity. On the basis of above facts, the effects of ambroxol and rebamipide are observed *in vitro* at the neutral pH of ER.

3.2. Materials and Methods

3.2.1. Animals

Charles-Foster strain of adult albino rats male (260 ± 20 g) was procured from Central Animal House; Institute of Medical Sciences, Banaras Hindu University (IMS-BHU) and acclimatized at a temperature of 25 ± 1^{0}C and 45-55% relative humidity with light/dark cycle of 12:12h by keeping them in polypropylene cages. Commercial food pellets (Doodhdhara Pashu Ahar, India) and water was made available *ad libitum.* No experiments were performed for one week in order to let the animals adapt to the laboratory conditions. All the experimental procedures were carried out in compliance with the principles of laboratory animal care [National Institutes of Health guide for the care and use of Laboratory animals (NIH Publication No. 8023, revised 1978)] guidelines and approved by the Institutional

animal ethical committee, BHU (Dean/2016/CAEC/33). The experiments were performed between 9:00h and 16:00h.

3.2.2. Materials

Ambroxol hydrochloride was received as a gift sample from Merril Pharma Pvt. Ltd. (Roorkee, India). Rebamipide was received as a gift sample from Akums Drugs & Pharmaceuticals Ltd., New Delhi, India. 4-methylumbelliferyl β-D-glucopyranoside, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES buffer, acid-free) and phenylmethylsulfonyl fluoride (PMSF), sucrose, mannitol, ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'- tetra acetic acid (EGTA), potassium dihydrogen phosphate (KH_2PO_4) and disodium hydrogen phosphate (Na_2HPO_4), were procured from Sigma-Aldrich (St. Louis, MO, USA). Sodium chloride (NaCl), potassium chloride (KCl), Tris-base, ethylenediamine tetraacetic acid disodium salt solution (EDTA), citric acid, dimethyl sulfoxide (DMSO), glycine, sodium hydroxide (NaOH) were acquired from Hi-media (Mumbai). All the remaining chemicals of HPLC (high-performance liquid chromatography) and analytical grades were acquired from local suppliers.

3.2.3. Isolation of endoplasmic reticulum (ER) from the brain region of rats

Isolation of ER-enriched fraction was carried out using previous described protocols with slight modifications (Hammond et al., 2012; Williamson et al., 2015). All steps were performed on ice, or at 4^0C for centrifugation steps. Three rats (n = 3) were killed by cervical dislocation and brain tissues were isolated. Tissues were washed using PBS 1X (140 mM NaCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 2.7 mM KCl pH 7.4). Tissues were pulverized and rewashed. Pulverized tissues (~1g) were

homogenized and reconstituted in 3.5 mL 1X ER extraction buffer [10 mM HEPES, 250 mM sucrose, 25 mM KCl and 1 mM EGTA, pH 7.8]. Just before use, protease inhibitor PMSF (100 mM) was added to the extraction buffer at a final concentration of 1 mM. A low speed centrifugation (700×g) of homogenate was performed for 10 minutes (min) in order to remove intact nuclei and large cellular debris (pellet 1). This is followed by the centrifugation of supernatant (supernatant 1) at 15000×g for 10 min to pellet intact mitochondria (pellet 2). The obtained supernatant (supernatant 2) was loaded onto a three-layered sucrose gradient [2.0 M sucrose (68.46 g sucrose, 10 mM Tris-base, 0.1 mM EDTA), 1.5 M sucrose (51.35 g sucrose, 10 mM Tris-base, 0.1 mM EDTA) and 1.3 M sucrose (44.50 g sucrose, 10 mM Tris-base, 0.1 mM EDTA), pH 7.6]. It was then centrifuged at 126000×g for 70 min on Beckman Coulter Optima™ MAX-XP ultracentrifuge. The resultant upper 200 mL of supernatant was taken out (supernatant 3). 100 to 300 mL of a dense white band between the top layer and the 1.3 M sucrose layer was collected (pellet 3). Pellet 3 was mixed by inversion with ice cold MTE buffer (270 mM D-mannitol, 10 mM Tris-base, 0.1 mM EDTA, pH 7.4) premixed with 200 mM PMSF buffer. This mixture was centrifuged at 126000×g for 45 min, producing a large, translucent pellet (pellet 4). Pellet 4 was reconstituted in 100 μL of PBS [pH 7.4] or assay buffer [pH 7]. All samples were stored in -20^{0}C for further use.

3.2.4. Effect of drugs on GCase activity *in vitro*

The effect of ambroxol and rebamipide was observed by using previously described protocol with slight modifications (Berger et al., 2015). Since GCase is synthesized in ER-bound polyribosomes and translocated to ER (pH 7) (Erickson et al., 1985;

Reczek et al., 2007), ER-enriched fraction was used as a source for GCase. The effect of drugs on GCase activity was determined at the neutral pH of ER. For IC50 (half maximal inhibitory concentration) and EC50 (half maximal effective concentration) experiments of drugs, assay buffer (McIlvaine Buffer), pH 7 was composed of 0.1M Citric acid (3.53 mL) and 0.2M sodium phosphate dibasic (16.47 mL). Drugs were dissolved in DMSO and diluted with assay buffer in order to make stock solution of 5000 μM. Further, dilutions were made to prepare 1000, 500, 100, 50, 10, 1, 0 μM solutions. ER-enriched fraction (final concentration of GCase 0.87 nM) was added to black 96-well plates in 10 μL volume, followed by addition of drug solutions (5 μL) and incubated for 30 min on ice. After incubation, assay buffer (pH 7, 100 μL) was added to the plate at a concentration of 4.65 mM. The substrate, 4-methylumbelliferyl β-D-glucopyranoside was freshly solubilized in distilled water at 37^0C prior to every experiment. The substrate was then added to the plate to a final concentration of 1.7 mM (25 μL). It was further incubated at 37^0C for 30 min, followed by addition of 75 μL stop solution (1 M glycine adjusted with 1M NaOH, adjusted to pH 10.5) to the plate. The experiment was performed in triplicate (n = 3) and the fluorescent signal was detected by using the EnVision Multilabel Plate Reader (Perkin Elmer, excitation = 355, emission = 450).

3.2.5. Statistical Analysis

IC50 and EC50 values were calculated using the software GraphPad Prism 5.01 and shown as mean ± standard deviation (SD).

3.3. Results & Discussion

The concepts of IC50 and EC50 are fundamental to pharmacology. EC50 is the concentration of a drug that gives half-maximal response. The IC50 is the concentration of an inhibitor where the response (or binding) is reduced by half (Sebaugh, 2011). The efficacy and potency of ambroxol and rebamipide was observed on GCase activity. In the graphs depicting % signal, vehicle treated (drug-free) samples were set as 100%. Ambroxol was found to be potent inhibitor of GCase as reported previously (Bendikov-Bar et al., 2011; Maegawa et al., 2009). GCase inhibition by ambroxol was dose-dependent and IC50 value was observed as 21.13 ± 10.39 µM at pH 7 of ER *in vitro*. Dose response curve of GCase inhibition by ambroxol is depicted in **Figure 3.1.** Rebamipide caused dose-dependent increase in GCase activity and EC50 value for GCase was observed to be 188.1 ± 63.23 µM. **Figure 3.2** depicts the dose response curve of GCase activation by rebamipide. GCase activating property of rebamipide at the pH of ER is observed for the first time in present study.

3.4. Conclusions

Ambroxol was found to be potent inhibitor of GCase at the pH of ER. The results were supported by earlier studies where ambroxol facilitate GCase-trafficking from ER to lysosome and get inactivated at lysosomal pH, thereby stimulating lysosomal GCase activity (Bendikov-Bar et al., 2011; Maegawa et al., 2009; Schapira, 2015). Rebamipide was observed as direct activator of GCase at the pH of ER, which is the novel finding. Both the drugs showed dose-dependent effects.

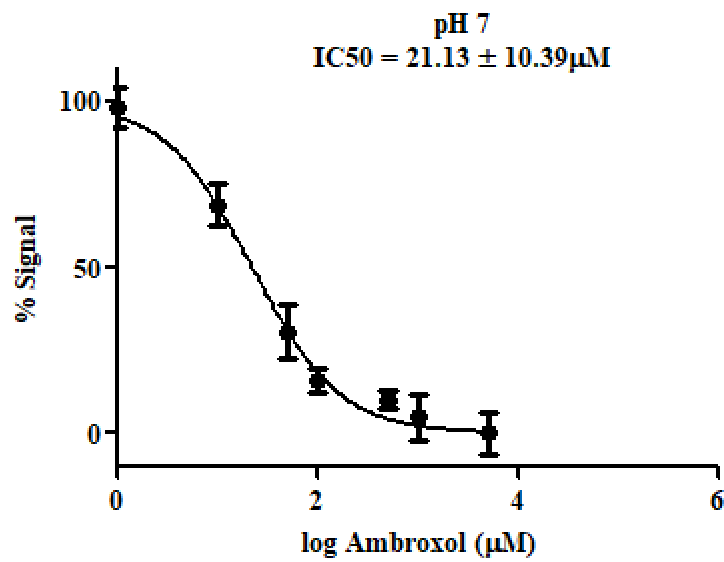

Figure 3.1 Dose response of GCase inhibition by ambroxol at pH 7; n = 3; IC50 values are shown as mean ± SD.

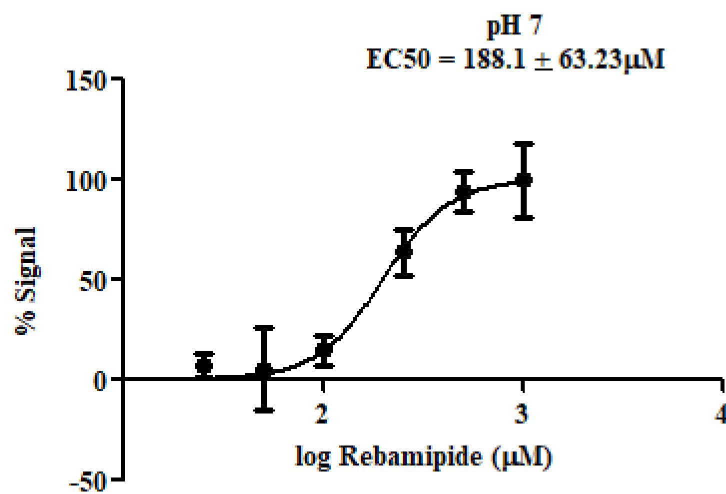

Figure 3.2 Dose response of GCase activation by rebamipide at pH 7; n = 3; EC50 values are shown as mean ± SD.