Ambroxol as a pharmacological chaperone for mutant glucocerebrosidase

Inna Bendikov-Bar a, Gali Maor a, Mirella Filocamo b, Mia Horowitz a,⁎

a Department of Cell Research and Immunology, Tel Aviv University, Ramat Aviv, 69978, Israel
b Centro di Diagnostica Genetica e Biochimica delle Malattie Metaboliche, IRCCS G. Gaslini, Genova, Italy

Abstract

Glucocerebrosidase (GCase) is synthesized on endoplasmic reticulum (ER)-bound polyribosomes, translocated into the ER and is subjected to ER quality control (ERQC), which senses the folding state of the newly synthesized proteins. Only properly folded molecules pass the ERQC and, following modifications and correct folding, shuttle to the lysosomes. Mutant GCase molecules, which fail to fold correctly, undergo ER associated degradation (ERAD) in the proteasomes, the degree of which is one of the factors that determine GD severity. Several pharmacological chaperones have already been shown to assist correct folding of mutant GCase molecules in the ER, thus facilitating their trafficking to the lysosomes. Ambroxol, a known expectorant, is one such chaperone. Here we show that ambroxol increases both the lysosomal fraction and the enzymatic activity of several mutant GCase variants in skin fibroblasts derived from Type 1 and Type 2 GD patients.© 2012 Elsevier Inc. All rights reserved.

Introduction

Gaucher disease (GD), the most common autosomal recessive lysosomal storage disorder is caused by mutations in the β-glucocerebrosidase (GCase) encoding gene GBA1. The disease is characterized by accumulation of glucosylceramide due to mutations in the GBA1 gene encoding the lysosomal hydrolase β-glucocerebrosidase (GCase). The disease has a broad spectrum of phenotypes, which were divided into three different Types; Type 1 GD is not associated with primary neurological disease while Types 2 and 3 are associated with central nervous system disease. GCase molecules are synthesized on endoplasmic reticulum (ER)-bound polyribosomes, translocated into the ER and following modifications and correct folding, shuttle to the lysosomes. Due to its heterogeneity, the disease has been subdivided into three clinical subtypes: the adult chronic non-neuronopathic Type 1 disease, the only Type in which patients do not present primary neurological signs (non-neuronopathic; MIM# 230800), the infantile, acute neuronopathic Type 2 disease (MIM# 230900) and the juvenile sub-acute neuronopathic Type 3 GD (MIM# 231000) [1] (For reviews see: [2–4]).

As a lysosomal enzyme, GCase is synthesized on endoplasmic reticulum (ER)-bound polyribosomes, translocated into the ER and is subjected to ER quality control (ERQC), which senses the folding state of the newly synthesized proteins. Only properly folded molecules pass the ERQC and, following modifications, are trafficked to lysosomes [5,6]. Mutant GCase molecules that fail to pass the ERQC are retained in the ER for further refolding attempts. After several unsuccessful attempts, the molecules are recognized as terminally misfolded and retrotranslocated from the ER to the cytoplasm, where they are degraded via the ubiquitin proteasome system. This whole process is called the ER associated degradation (ERAD). Mutant GCase variants present variable degrees of ER retention and undergo ERAD, the degree of which is one of the factors that determine GD severity [7–9].

The current standard of care for individuals with GD [4] is enzyme replacement therapy (ERT), in which intravenous infused recombinant enzyme is internalized by monocyte derived cells via mannose receptors and is transported to lysosomes, to break down accumulated substrate [10]. The current ERT for GD is limited to the treatment of non-neurological symptoms, due to the inability of the enzyme to cross the blood–brain barrier [3]. Another therapy, known as substrate reduction therapy (SRT), uses glucosyl-ceramide synthase inhibitor as a means to lower the amount of accumulated substrate, thus lowering the level of residual activity necessary to prevent storage [11] SRT has had some success at ameliorating clinical signs.

Pharmacological chaperone therapy (PCT) is another emerging strategy for treatment of GD patients. Pharmacological chaperones are small molecules that are able to bind misfolded proteins in the ER and assist their folding, thus enabling them to pass the ERQC and shuttle to the lysosomes. Due to low pH and high substrate concentration in the lysosomes, the enzyme–chaperone complexes are dissociated and the mutant enzyme, depending on its residual activity, hydrolyzes its natural substrate [12].

There is a vast amount of publications on the use of chaperones to enhance lysosomal GCase activity [13–23]. One such chaperone is ambroxol [24–26]. Addition of ambroxol to tissue culture fibroblasts increased the GCase activity in cells with the genotypes: N370S/N370S and F213I/L444P [24], L444P/L444P [9], N188S/G193W and R120W [25]. There are two major advantages of using ambroxol as a pharmacological chaperone for Gaucher disease. Firstly, it has a long history of use.
in humans, which documents its very low level of toxicity. Secondly, binding of ambroxol to GCase is highly pH dependent. It binds best at the neutral pH of the ER where it helps with folding, and poorest at the acidic pH of the lysosome, where it is no longer needed [24]. In the present report we extended our previous study and document elevation in the lysosomal fraction and enzymatic activity of five additional mutant GCase variants by Ambroxol.

Materials and methods

Materials

The following antibodies were used in this study: monoclonal anti-GCase 2E2 (Sigma), rabbit polyclonal anti-GCase (C4171, Sigma), rabbit polyclonal anti-calnexin (Sigma), rabbit anti-erk (C16 Santa Cruz Biotechnology, Santa Cruz, CA, USA); secondary antibodies: horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit were purchased from Jackson Immuno Research Laboratories (West Grove, PA, USA).

Four-methyl-umbelliferyl-glucopyranoside (4-MUG) was from Sigma-Aldrich, Israel. Nonident P-40 (NP-40) was from Roche Diagnostic (Mannheim, Germany). Leupeptin was from Sigma-Aldrich, Israel. Endonuclease-H (Endo-H) was purchased from New England Biolabs (Beverly, MA, USA).

Cell lines

Human primary skin fibroblasts, detailed in Table 1, were grown in DMEM, supplemented with 20% FCS (Beit Haemek, Israel) at 37 °C in the presence of 5% CO₂.

Endonuclease-H (Endo-H) treatment

Samples of cell lysates, containing 80 µg of total protein, were subjected to an overnight incubation with Endo-H, according to the manufacturer’s instructions.

Ambroxol treatment

Sub-confluent human skin fibroblasts, grown on 9 mm plates, were treated with different concentrations of ambroxol hydrochloride (A9797, Sigma-Aldrich). Twenty hours later, cells were washed three times with ice-cold phosphate-buffered saline (PBS) and lysed at 4 °C in 150 µl of lysis buffer (10 mM HEPES pH 8.0, 100 mM NaCl, 1 mM MgCl₂ and 1% Triton X-100) containing 10 mg/ml aprotinin, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 mg/ml leupeptin. Samples containing the same amount of protein, as determined by the Bradford technique [27], were subjected to western blot analysis.

Enzymatic activity

Confluent primary skin fibroblasts were washed twice with ice-cold PBS and collected in 150 µl sterile water. Forty micrograms of the lysates were assayed for GCase activity in 0.2 ml of 100 mM potassium phosphate buffer, pH 4.5, containing 0.15% Triton X-100 (v/v, Sigma, Israel) and 0.125% taurocholate (w/v, Calbiochem, La Jolla, CA, USA) in the presence of 1.5 mM 4-MUG for 1 h at 37 °C. The reaction was stopped by the addition of 0.5 ml of stop solution (0.1 M glycine, 0.1 M NaOH, pH 10). The amount of 4-methyl-umbelliferone (4-MU) was quantified using Perkin Elmer Luminescence Spectrometer LS 50 (excitation length: 340 nm; emission: 448 nm).

Quantitation

The blots were scanned using Image Scan scanner (Amersham Pharmacia Biotech, GE Healthcare, Buckinghamshire, UK) and the intensity of each band was measured by the image master densitometer 1Dprime (Amersham Pharmacia Biotech, GE Healthcare, Buckinghamshire, UK).

Table 1

| Genotype | Disease type | Lysosomal fraction of GCase |
|----------|--------------|----------------------------|
|          |              | Ambroxol conc. (µM)         |
|          |              | 0  | 10  | 25  | 50  | 100 |
| Normal   | Normal       | 89.1 | 96.6 | 85.7 | 93.8 | 90.4 |
| N370S/V394L | 1           | 40.8 | 50.5 | 52.6 | 53.5 | 54.5 |
| N370S/N370S | (severe)   | 22.6 | 31.0 | 31.0 | 30.7 | 28.3 |
| L444P/L444P | 3           | 10.4 | 18.6 | 25.6 | 23.1 | 57.6 |
| L444P/L444P | (severe)    | 3   | 7.2  | 7.2  | 11.4 | 15.3 | 12.4 |
| L444P/R120W | 2           | 8.9  | 7.2  | 6.7  | 7.0  | 8.3  |
| L444P/P415R | 2           | 8.4  | 11.2 | 21.3 | 30.5 | 51.1 |
| R131C/R131C | 2           | 4.5  | 7.3  | 20.7 | 39.0 | 22.4 |

The lysosomal fraction of GCase was calculated by multiplying the endo-H resistant fraction with the total GCase in the same lane (compared to normal, which is considered 100), divided by 100. The resulted value reflects the amount of GCase in lysosome as a percent of total normal GCase. The Endo-H resistant fraction in the different cell lines, as well as total GCase, were calculated as explained in the legend of Fig. 2. 1,2,3 cell lines were presented in [9].

Fig. 1. Effect of ambroxol on GCase activity. Skin fibroblasts from normal and five GD patients (type of disease appears in parenthesis) were treated with increasing concentrations of ambroxol for 20 h at 37 °C. Samples containing 40 µg of protein were tested for GCase activity using 1.5 mM of the artificial substrate 4-MUG. Data are expressed as fold increase of GCase activity in the presence of ambroxol in comparison to untreated cells. The results represent the mean ± SEM, of three to five independent experiments.
Results

Ambroxol enhances enzymatic activity and lysosomal localization of mutant GCase variants

We evaluated the effects of ambroxol treatment on residual GCase activity, as well as on processing and trafficking of several previously untested mutant GCase variants.

Treatment of skin fibroblasts that originated from Gaucher patients, with increasing concentrations of ambroxol resulted in 15–50% increase in activity of the mutant protein in all five tested lines (Fig. 1).

We used Endo-H sensitivity as a measure of GCase trafficking from the ER to the lysosomes, as we have previously done [7], and calculated the increase in lysosomal GCase as a function of increase in ambroxol concentration. As presented in Table 1 and Fig. 2, ambroxol treatment increased the amount of mutant GCase in the lysosomes of all tested lines (black bars, Fig. 2). The increase in the amount of lysosomal GCase in cells that derived from Type 1 GD patients (genotypes: N370S/N370S, N370S/V394L) was accompanied by an elevation in enzymatic activity, strongly suggesting that mutant enzymes had activity in the lysosomes. In two Type 2 derived skin fibroblasts (genotypes: R120W/L444P, P415R/L444P), treatment with ambroxol resulted in a significant elevation in lysosomal mutant GCase protein fraction and a

![Fig. 2. Effect of Ambroxol on total GCase amount and the lysosomal fraction of GCase. Skin fibroblasts from an unaffected individual (A) and five GD patients (B–F) were treated with increasing concentrations of ambroxol for 20 h at 37 °C. Samples containing 100 μg of protein, were subjected to Endo-H digestion overnight, electrophoresed through SDS-PAGE and the corresponding blot was interacted with anti GCase and anti erk antibodies. To normalize the results the blots were scanned and the intensity of each band was measured. To determine the amount of total GCase, the intensity of the GCase band at each lane was divided by the intensity of the erk at the same lane. The value obtained for the normal untreated cells was considered as 100%. To determine the Endo-H resistant fraction, the intensity of GCase resistant fraction was divided by the intensity of the entire amount of GCase in the same lane. The amount of GCase in lysosomes was calculated by multiplying the Endo-H resistant fraction with the total GCase in the same lane (compared to normal, which was considered 100), divided by 100. The results represent the mean of three to five independent experiments.](image-url)
concomitant increase in GCase activity. Treatment of cells that originated from a Type 2 GD patient with the genotype R131C/R131C, which had very low lysosomal GCase amount, led to both an increase in lysosomal GCase protein fraction and an increase in GCase activity. The presented results indicate that ambroxol not only removes the mutant protein from the ER, after which it shuttles to the lysosomes, but also prevents its degradation and in some cases increase the lysosomal activity of mutant GCase variants.

Discussion

The results presented in this study confirm previous reports that documented the ability of ambroxol to enhance folding of mutant GCase in the ER and to assist shuttling of the latter to the lysosomes. This leads to increased amounts of lysosomal GCase protein and concomitant increase in enzymatic activity, depending on the nature of the mutation.

Lysosomal storage diseases in general and Gaucher disease in particular are good candidates for pharmacological chaperone therapy. The first pharmacological chaperones for mutant GCase were members of the iminosugar family, naturally occurring glycosidase inhibitors. Their structure resembled that of natural glycosides, except for the presence of a nitrogen atom in place of the endocyclic oxygen of the substrate [28]. The first molecule reported as pharmacological chaperone for GCase was the iminosugar N-(n-Nonyl) 1-deoxynojirimycin, (NN-DNJ), which increased the activity of the N370S mutant GCase variant [29]. The iminosugar isophagolamine was shown to increase residual GCase activity in fibroblasts that originated from Gaucher patients with different genotypes [30,31], as well as in mice homozygous for the GCase mutations: V394L, D409H, or D409V [32]. For a review on more competitive inhibitors see refs [33,34].

The response to pharmacological chaperones seems to be patient dependent; therefore the identification of more potential pharmacological chaperones for mutant GCase variants remains a subject for extensive research. High throughput screens of small molecule libraries have led to identification of novel chaperone molecules [14,18,19,22,23,35,36]. Another approach is the synthesis of inhibitors, based on the structure of known potential chaperones. Recently, such molecules were reported to increase the enzymatic activity of the N370S and G202R mutant GCase variants [13,17,20].

Ambroxol, a well-known expectorant that was used to treat airway infections in humans, was isolated in a screen of an FDA approved small molecule library, having the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248.

Acknowledgments

We would like to thank Prof E Shprecher (Department of Dermatology, Tel Aviv Sourasky Medical Center, Tel Aviv, Israel) for providing healthy skin fibroblasts and Prof EH Kolodny (NYU Medical Center, NY, USA) for the Gaucher patients cell lines. We also thank the “Cell Line and DNA Biobank from Patients Affected by Genetic Diseases” (G. Gaslini Institute), Telthon Genetic Biobank for GD skin fibroblasts. This work was supported by grants from the Israel Science Foundation and the Israeli Ministry of Health (to MH) and the Telthon Genetic Biobank Network Project No. GTB07001A (to MF).

References

[1] R.O. Brady, N.W. Barton, G.A. Grabowski, The role of neurogenetics in Gaucher disease, Arch. Neurol. 59 (3) (1992) 1212–1224.
[2] E. Beutler, Gaucher disease, Adv. Genet. 32 (1995) 17–49.
[3] G.A. Grabowski, Phenotype, diagnosis, and treatment of Gaucher’s disease, Lancet 372 (2008) 1263–1271.
[4] I.S. Bonifacino, A.M. Weissman, Ubiquitin and the control of protein fate in the secretory and endocytic pathways, Annu. Rev. Cell Dev. Biol. 14 (1998) 19–57.
[5] I.R. Kopito, ER quality control: the cytoplasmic connection, Cell 88 (1997) 427–430.
[6] Z. Luan, R. Horowitz, ER retention and degradation as the molecular basis underlying Gaucher disease heterogeneity, Hum. Mol. Genet. 14 (2005) 2387–2388.
[7] Z. Luan, R. Horowitz, Intracellular cholesterol modifies the ERAD of glucocerebrosidase in Gaucher disease patients, Mol. Genet. Metab. 93 (2008) 426–436.
[8] Z. Luan, H. Lian, E. Goldin, M. Jeyakumar, High throughput screens of small molecule chaperones for the treatment of Gaucher disease, J. Med. Chem. 55 (2012) 6857–6865.
[9] Z. Luan, E. Goldin, M. Jeyakumar, Substrate reduction therapy, Acta Paediatr. Suppl. 97 (2008) 86–93.
[10] E. Beutler, Enzyme replacement in Gaucher disease, PLoS Med. 1 (2004) e21.
[11] F.M. Platt, M. Jeyakumar, Substrate reduction therapy, Acta Paediatr. Suppl. 97 (2008) 86–93.
[12] R.L. Lieberman, J. Castilla, E. Beutler, E. Goldin, J. Marugan, Non-inhibitory small molecule chaperones, ChemMedChem 6 (2) (2011) 56–60.
[13] G. Babajani, et al., Pharmacological chaperones facilitate the post-ER transport of glucocerebrosidase variant, Blood Cells Mol. Dis. 46 (2011) 1–4.
[14] E. Goldin, et al., High throughput screening for small molecule therapy for Gaucher disease using patient tissue as the source of mutant glucocerebrosidase, PLoS One 7 (2012) e29861.
[15] Z. Luan, et al., A Fluorescent sp2-iminosugar with pharmacological chaperone activity for gaucher disease: synthesis and intracellular distribution studies, Chembiochem 11 (17) (2010) 2453–2464.
[16] J.J. Marugan, et al., rapid assembly of a library of lipophilic iminosugars for the treatment of Gaucher disease, ChemMedChem 6 (2) (2011) 353–361.
[17] E.D. Goddard-Borger, et al., Binding of 3,4,5,6-tetrahydroxyazepanes to the acid-beta-glucosidase active site: implications for pharmacological chaperone design for Gaucher disease, Biochemistry 50 (2011) 10647–10657.
[18] S. Patnaik, et al., Discovery, structure–activity relationship, and biological evaluation of noniminosugar small molecule chaperones of glucocerebrosidase, J. Med. Chem. 55 (2012) 5734–548.
[19] A. Trapero, et al., Potent aminocyclitol glucocerebrosidase inhibitors are subnanomolar pharmacological chaperones for treating gaucher disease, J. Med. Chem. 55 (2012) 4479–4488.
[20] G.N. Wang, et al., Synthesis of N-substituted epsilon-hexanolactams as pharmacological chaperones for the treatment of N370S mutant Gaucher disease, Org. Biomol. Chem. 10 (2012) 2923–2927.
[21] E.D. Goddard-Borger, et al., Rapid assembly of a library of lipophilic iminosugars via the thio-ene reaction yields promising pharmacological chaperones for the treatment of Gaucher disease, J. Med. Chem. 55 (2012) 2737–2745.
[22] J.J. Marugan, et al., Non-iminosugar glucocerebrosidase small molecule chaperones, MedChemComm 3 (2012) 56–60.
[23] W. Zheng, et al., Three classes of glucocerebrosidase inhibitors identified by quantitative high-throughput screening are chaperone leads for Gaucher disease, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 13192–13197.
[24] G.H. Maegawa, et al., Identification and characterization of ambroxol as an enzyme enhancement agent for Gaucher disease, J. Biol. Chem. 284 (2009) 23502–23516.
[25] Z. Luan, et al., The chaperone activity and toxicity of ambroxol on Gaucher cells and normal mice, Brain Dev. (2012) [Electronic publication ahead of print].
[26] C. Babajani, et al., Pharmacological chaperones facilitate the post-ER transport of recombinant N370S mutant beta-glucocerebrosidase in plant cells: evidence that N370S is a folding mutant, Mol. Genet. Metab. 106 (2012) 323–329.
[27] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[28] L. Colombeau, et al., Metal-free one-pot oxidative amidation of aldoxides with functionalized amines, J. Org. Chem. 73 (2008) 8647–8650.
[29] A.R. Sawkar, et al., Chemical chaperones increase the cellular activity of N370S beta-glucosidase: a therapeutic strategy for Gaucher disease, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 15428–15433.

[30] H.H. Chang, et al., Hydrophilic iminosugar active-site-specific chaperones increase residual glucocerebrosidase activity in fibroblasts from Gaucher patients, FEBS J. 273 (2006) 4082–4092.

[31] R.A. Steet, et al., The iminosugar isofagomine increases the activity of N370S mutant acid beta-glucosidase in Gaucher fibroblasts by several mechanisms, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 13813–13818.

[32] Y. Sun, et al., Ex vivo and in vivo effects of isofagomine on acid beta-glucosidase variants and substrate levels in Gaucher disease, J. Biol. Chem. 287 (2012) 4275–4287.

[33] Z. Yu, et al., Isofagomine- and 2,5-anhydro-2,5-imino-D-glucitol-based glucocerebrosidase pharmacological chaperones for Gaucher disease intervention, J. Med. Chem. 50 (2007) 94–100.

[34] J.M. Benito, J.M. Garcia Fernandez, C. Ortiz Mellet, Pharmacological chaperone therapy for Gaucher disease: a patent review, Expert Opin. Ther. Pat. 21 (2011) 885–903.

[35] W. Huang, et al., N4-phenyl modifications of N2-(2-hydroxyl)ethyl-6-(pyrrolidin-1-yl)-1,3,5-triazine-2,4-diamines enhance glucocerebrosidase inhibition by small molecules with potential as chemical chaperones for Gaucher disease, Bioorg. Med. Chem. Lett. 17 (2007) 5783–5789.

[36] M.B. Tropak, et al., Identification of pharmacological chaperones for Gaucher disease and characterization of their effects on beta-glucocerebrosidase by hydrogen/deuterium exchange mass spectrometry, ChemBioChem 9 (2008) 2650–2662.

[37] B. Rigat, D. Mahuran, Diltiazem, a L-type Ca(2+) channel blocker, also acts as a pharmacological chaperone in Gaucher patient cells, Mol. Genet. Metab. 96 (2009) 225–232.

[38] E.R. Benjamin, et al., Co-administration with the pharmacological chaperone AT1001 increases recombinant human alpha-galactosidase A tissue uptake and improves substrate reduction in Fabry mice, Mol. Ther. 20 (2012) 717–726.

[39] C. Porto, et al., Synergy between the pharmacological chaperone 1-deoxygalactonojirimycin and the human recombinant alpha-galactosidase A in cultured fibroblasts from patients with Fabry disease, J. Inherit. Metab. Dis. 35 (2012) 513–520.