



In vitro and *in vivo* effects of Ambroxol chaperone therapy in two Italian patients affected by neuronopathic Gaucher disease and epilepsy

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ABSTRACT

Gaucher disease (GD) is an autosomal recessive lysosomal storage disorder caused by mutations in the acid β -glucosidase encoding gene (*GBA1*), resulting in the deficient activity of acid β -glucosidase (GCCase). To date, there is no approved treatment for the neurological manifestations of the disease. The role of Ambroxol as a chaperone for mutant GCCase has been extensively demonstrated *in vitro*. Furthermore, different authors have reported beneficial effects of high doses of Ambroxol on neurological manifestations in patients affected by GD.

In this report, we describe the *in vitro* and *in vivo* effects of Ambroxol in two patients (P1 and P2) affected by the neurological form of GD and epilepsy, carrying mutations already reported as responsive to the chaperone. Indeed, P1 presented the N188S mutation in compound heterozygous with a null allele (IVS2 + 1G > A) and P2 was homozygous for the L444P mutation. As expected, a beneficial effect of Ambroxol was observed in cultured fibroblasts as well as *in vivo*, both on epilepsy and on biomarkers of GD, in P1. However, Ambroxol was completely undetectable in P2, suggesting that other factors besides the *GBA1* mutation itself would be involved in the response therapy which would be difficult to predict based on the patient genotype. The present report expands the experience of Ambroxol treatment in neurological GD patients and highlights the need to *in vitro* test the individual response to Ambroxol even in patients carrying mutations already classified as responsive to the chaperone.

1. Introduction

Gaucher disease (GD) is an autosomal recessive lysosomal storage disorder caused by mutations in the acid β -glucosidase encoding gene (*GBA1*), resulting in the deficient activity of acid β -glucosidase (GCCase), and the subsequent progressive accumulation of glucosylceramide within the lysosomes [1].

The presence, severity and rate of progression of neurological involvement are discriminating factors for GD classification into three different clinical phenotypes, although the clinical picture presents as a phenotypic continuum. Type-1 GD, the most frequent phenotype, is the non-neuronopathic form of GD, whereas type-2 GD and type-3 GD are

collectively referred as neuronopathic GD (nGD), representing the acute and chronic neuronopathic phenotypes, respectively [2].

Ambroxol, a common drug which has been used for decades as a mucolytic agent, has been shown to act as a pharmacologic chaperone for mutant GCCase [3]. As other pharmacological chaperones (PC), Ambroxol is able to bind mutant misfolded GCCase proteins at neutral pH in the endoplasmic reticulum (ER) and promote their correct folding, enabling the mutated proteins to escape the endoplasmic reticulum associated degradation (ERAD) and to reach the lysosome. Low pH values and high concentration of substrate within the lysosome favor the release of the enzyme from the chaperone complex allowing it to hydrolyze the substrate, if the mutant protein is partially active [3].

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In vitro, the efficacy of Ambroxol has been shown in fibroblast from GD patients displaying different genotypes (N370S/N370S, F2131/L444P, L444P/L444P, N188S/G193W, F213/L444P, F213/RecNeil, R131C/R131C, and R120W) [3–6].

A pilot study using Ambroxol (2 capsules of 75 mg daily for 6 months) in twelve naive patients affected by type-1 GD, resulted in individual improvement of disease parameters (anemia/thrombocytopenia, spleen and liver volume) [7]. This molecule crosses the blood brain barrier and it was shown to enhance endogenous GCase activity in the central nervous system (CNS) of normal mice and healthy nonhuman primate [5,8]. Based on these findings Ambroxol was proposed for the treatment of type-3 GD patients. A pilot study in five patients affected by nGD, demonstrated that high doses of oral Ambroxol (25 mg/Kg/day or a maximum of 1300 mg/day) resulted in markedly improvement of myoclonus, seizures and pupillary light reflex dysfunction in all patients [9]. This positive clinical experience on epilepsy was confirmed in several type-3 GD patients [10,11].

In this report, we describe the *in vitro* and *in vivo* effects of a high-dose Ambroxol in two patients affected by nGD and epilepsy, carrying two different *GBA1* genotypes.

2. Methods

2.1. Cell culture and Ambroxol treatment

Fibroblasts from two patients affected by type-3 GD were cultured and maintained in Dulbecco's modified Eagle's medium High Glucose (EuroClone) containing 10% fetal bovine serum (Gibco), 1% glutamine (Gibco), and 1% penicillin/streptomycin (Gibco), in a humidified atmosphere containing 5% CO₂ at 37 °C.

Fibroblasts, grown on 100 mm plates, were treated with Ambroxol hydrochloride (A9797, Sigma-Aldrich) 100 μM dissolved in Dimethyl Sulfoxide 0,1% (DMSO – Santa Cruz Biotechnology) or with an equal volume of vehicle (DMSO), as control [6]. Twenty-two hours later, cells were lysed in water and sonicated. Total amount of protein in cell lysates was determined by Bradford assay, using the Biorad-Protein Assay (BioRad), following manufacturer's instructions.

2.2. Enzymatic activity

GCase enzymatic activity was measured using the fluorogenic substrate 4-methylumbelliferyl-β-D-glucopyranoside (Sigma M3663 – PM 338.31). Briefly, 10 μl containing 10 μg of protein were incubated with 10 μl of substrate 5 mM in acetate buffer 0.1 M pH 4.2 at 37 °C for 3 h. Reaction was stopped with carbonate buffer 0.5 M pH 10.7 and the fluorescent product was quantified using a fluorimeter (SPECTRAMax Gemini XPS, Molecular Devices) at excitation wavelength of 365 nm and emission of 495 nm.

Chitotriosidase activity was assessed in serial plasma samples of P1 using the 4-MU-β-D-triacetylchitotriosidase (Sigma, St. Louis, MO, USA) as a substrate.

2.2.1. Glucosylsphingosine measurement (Lyso-Gb1)

Lyso-Gb1 measurement was performed as previously described [12]. Briefly, after plasma purification by combination of protein precipitation, evaporation and reconstitution in mobile phase, reverse-phase liquid chromatography was performed using a Shimadzu Nexera XR UPLC (Shimadzu, Kyoto, Japan) and a Poroshell 120 EC-C8 column, 3.0 × 50.0 mm with 2.7 μm particle size (Agilent, Santa Clara, USA). Mass spectrometry detection was carried out with AB Sciex 4000 QTrap tandem mass spectrometer (Sciex, MA, USA) set in positive mode using an electrospray ionization (ESI) source [12].

2.2.2. Molecular diagnosis

Molecular analysis: Genomic DNA was extracted from peripheral blood leukocytes, cultured fibroblasts and lymphoblasts using QIAamp

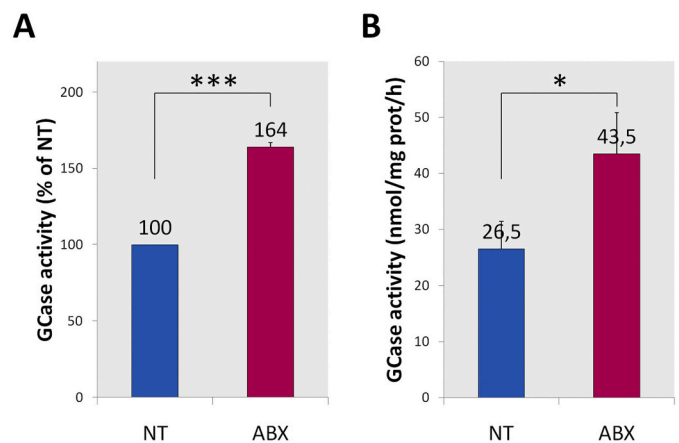


Fig. 1. GCase activity in P1 fibroblasts.

Fibroblasts were treated with Ambroxol hydrochloride 100 μM (ABX) or with an equal volume of vehicle (0,1% DMSO; NT). Values are expressed as the percentages of each NT patient (A) or as nmol/mg protein/h (B). Results are expressed as mean ± SD of three independent experiments, each performed in duplicate. * $p < 0.05$, *** $p < 0.0001$.

DNA blood Mini Kit (Qiagen GmbH, Hilden, Germany) or Nucleon BACC3 kit (Amersham Biosciences, Buckinghamshire, UK). *GBA1* gene exons and most intronic regions were PCR amplified using primers designed by reference to the genomic sequence (GenBank J03059.1) to selectively amplify the gene and not the homologous pseudogene (GenBank J03060.1) as previously described [13].

PCR products were analyzed by automated sequencing (ABI Prism 3500xl genetic analyzer, Applied Biosystems, Foster City, CA, USA). Putative mutations were confirmed by sequencing duplicate PCR products and by the DNA analysis from parents.

2.2.3. Treatment

FLUIBRON® Ambroxolo (CHIESI FARMACEUTICI S.p.A.) was commercially purchased as 30 mg Ambroxol hydrochloride tablets. Oral Ambroxol was administered at the target dose (25 mg/kg/day or a maximum dose of 1300 mg/day).

2.3. Statistical analysis

Statistical analysis was performed using Student's *t*-test. $P < 0,05$ was considered as statistically significant.

3. Results

Patient 1 (P1) is a girl diagnosed with type-3 GD at the age of 6 years, carrying the *GBA1* IVS2 + 1G > A mutation in compound heterozygosity with the N188S pathogenic variant, strongly associated with myoclonic epilepsy [14,15]. The patient presented with moderate peripheral involvement and at the time of diagnosis her neurological examination was normal, as well as instrumental investigations (EEG, IQ, brain MRI).

Enzyme replacement therapy (ERT) was started immediately at the dose of 60 Units/Kg every other week; visceral and hematological parameters normalized quickly. Chitotriosidase progressively decreased from 929 nmol/ml/h (before treatment) to 272 nmol/ml/h (after 3 years on ERT) and then remained stable, while plasma Lyso-Gb1 decreased from 31.4 ng/ml before treatment to 9.9 ng/ml after 3 years of treatment and then remained stable. During the follow up, the EEG became increasingly pathologic; at age 14 she started to have frequent episodes of generalized seizures with a duration of about 2 min, always in the morning during awakening or during the pre-menstrual period. Ocular abnormalities, *i.e.* pathological saccadic movements, were highlighted,

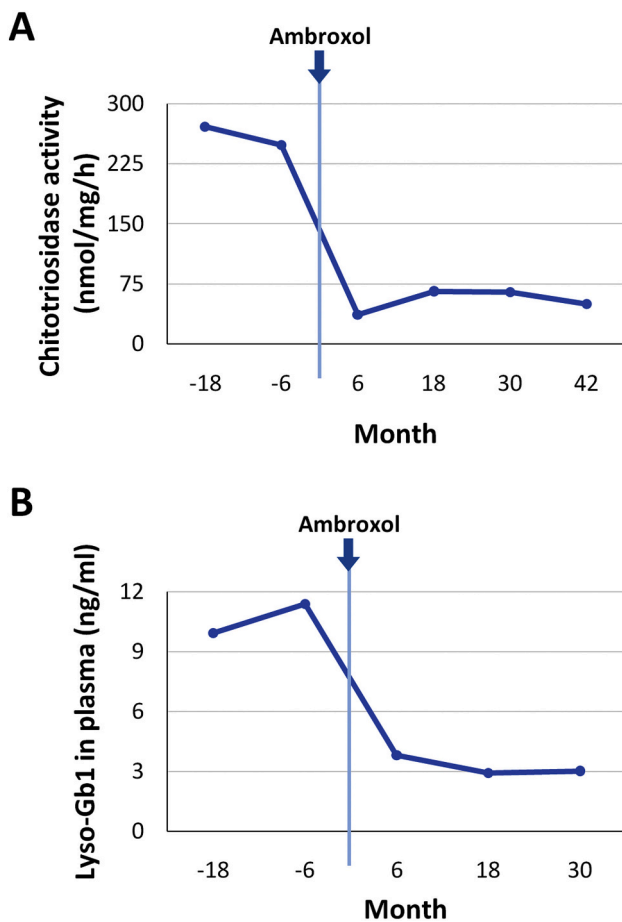


Fig. 2. Plasma levels of Gaucher biomarkers in P1.

A) Chitotriosidase activity (nmol/mg/h); B) Lyso-Gb1 levels (ng/ml)

while IQ remained normal. She started therapy with sodium valproate, levetiracetam and clonazepam. With this treatment, seizures frequency decreased, but 2–3 episodes/week with same duration would still happen despite the increase of the levetiracetam dose.

At that point we decided to test the possible effect of Ambroxol *in vitro*. The residual enzymatic activity in untreated cultured fibroblasts was 26.5 nmol/mg/h (normal value: 86.12 ± 25.00), which increased to 43.5 nmol/mg/h (67% increase) after 24 h treatment (Fig. 1).

Therefore, at the age of 14 years and 6 months she began Ambroxol treatment at 20 mg/Kg/day in 2 doses and then, after 12 months, at 25 mg/Kg/day in 3 doses.

Chitotriosidase activity and Lyso-Gb1 levels decreased after 6 months of treatment from 247.5 nmol/ml/h to 37.2 nmol/ml/h and from 11.4 ng/ml to 3.8 ng/ml, respectively (Fig. 2).

Frequency of seizures slowly decreased and after 30 months of treatment, at age 17, she reported only one seizure crisis/month of short duration (less than 1 min), always in the morning during awakening. Saccadic movements remained pathological.

At age 18, after 42 months of treatment, she reported no seizure crisis in the last 6 months. In the meantime, she had stopped levetiracetam and clonazepam, 6 and 5 months before respectively. In addition, sodium valproate had been decreased from 500 mg twice daily to 300 mg in the morning +500 mg in the evening in the last month. Saccadic movements remained unchanged. No side effects were observed during Ambroxol hydrochloride treatment period. A summary of the evolution of neurological involvement is shown in Table 1.

Patient 2 (P2) is a man diagnosed with type-3 GD at age 14. When he was 16 years old, he developed epilepsy, defined as “cryptogenetic”, characterized by brief impaired awareness at spontaneous resolution, coupled to tonic-clonic jerks of the limbs. It was defined a drug resistant epilepsy. Molecular diagnosis showed the presence of the common L444P mutation in homozygosity. He started ERT in 1995, when he was 37 years old, at a dose of 60 Units/kg/every other week without any effect on critical episodes. At that point, epilepsy was treated with vigabatrin, oxcarbazepine and phenobarbital. The patient had a concomitant mild mental retardation (Wechsler Adult Intelligent Scale –Revised, total IQ 70). Neurological examination showed a mild camptocormia with a slight wide-based gait, possible both on toes and on heels, difficult in tandem. The remaining neurological examination was otherwise normal. Ocular movements assessment was not performed, due to right eye retinal detachment at age 30, with secondary glaucoma in the same eye and severe myopia.

At age 60, based on his poor neurological clinical outcome, the possibility to start Ambroxol treatment was evaluated. *In vitro* studies showed that the residual enzymatic activity in patient’s cultured fibroblasts was 12.8 nmol/mg/h (normal value: 86.12 ± 25.00), and no effect of Ambroxol was observed after 24 h treatment (Fig. 3).

Despite this negative result, due to the worsening of the neurological involvement he began oral treatment with Ambroxol, that was gradually titrated to a maximum dose of 1300 mg/daily administered in 3 divided doses. Four months after the maximum dose was reached, no effects were observed in the frequency of the critical episodes. Camptocormia and gait remained stable and the EEG graphic was unchanged. The chitotriosidase level at baseline was 317.92 nmol/ml/h and did not change significantly after 12 months. Lyso-Gb1 levels were not available. We discussed the clinical issue with the patient and his relatives, and it was agreed to stop Ambroxol therapy.

Except for mild abdominal discomfort when taking the higher dose, no other side effects were observed. A summary of the neurological involvement is shown in Table 1.

4. Discussion

In this report we describe the effect of high doses of Ambroxol (25 mg/Kg/day and 1300 mg/day, respectively) in two GD patients with different genotypes. Treatment had different effects on epilepsy and biochemical markers (chitotriosidase and Lyso-Gb1), being effective in P1 patient and ineffective in P2. Although in P2 the highest dose of Ambroxol was given for a period significantly shorter than for P1, the absence of clinical improvement and the lack of Ambroxol effect on residual enzymatic activity *in vitro*, prompted us to interrupt treatment.

Indeed, the clinical response to Ambroxol in these two patients was in line with the results obtained by *in vitro* studies.

Treatment of P1 fibroblasts with Ambroxol resulted in 67% increase of enzymatic activity while no response to Ambroxol was observed in fibroblasts from P2. Regarding the genotype, P1 carries the N188S mutation in compound heterozygosity with a splicing mutation leading to the lack of mRNA expression. Our results are in agreement with those reported in the literature showing that the N188S mutation was responsive to Ambroxol [4,9]. Moreover, this mutation was also present in three out of four neuronopathic Gaucher patients reported by Narita et al. [9], who clinically responded to Ambroxol treatment in terms of epileptic crisis reduction.

Conversely, treatment of P2 fibroblasts had no effect on GCase residual activity. P2 is homozygous for the L444P mutation, the most frequent mutation identified in nGD patients. *In vitro* studies on the effect of Ambroxol on GCase activity in fibroblasts from patients carrying this mutation have shown variable results [3,4,16]. This suggests that

Table 1
Evolution of Neurological Involvement.

Age (year)	Neurological involvement pre-therapy	Therapy	Neurological involvement post-therapy	Side effects
Patient 1				
6	Diagnosis of GD	ERT was started (60 Units/Kg every other week).		
14	<ul style="list-style-type: none"> Neurological examination normal EEG became increasingly pathologic. Generalized seizures (duration of about 2 min, in the morning during awakening or during the pre-menstrual period). Ocular abnormalities, i.e. pathological saccadic movements. 	Sodium valproate (500 mg twice daily), levatiracetam (500 mg twice daily) and clonazepam (1 mg/day) were started.	Seizures frequency decreased, but 2–3 episodes/week with same duration still occurred	
14.5		Ambroxol treatment was started (20 mg/Kg/day in 2 doses).	Frequency of seizures slowly decreased	
15.5		Increased Ambroxol treatment (25 mg/Kg/day in 3 doses).	Frequency of seizures slowly decreased	
17		Unchanged Ambroxol treatment	Only one seizure crisis/month of short duration (< 1 min), always in the morning during awakening. Saccadic movements remained pathological.	
18		Sodium valproate was decreased (300 mg in the morning +500 mg in the evening) in the last month. Both levatiracetam and clonazepam were stopped, 6 and 5 months before, respectively.	No seizure crisis in the last 6 months. Saccadic movements remained unchanged. IQ remained normal	No side effects during Ambroxol treatment
Patient 2				
14	Diagnosis of GD			
16	Neurological examination: normal. "Cryptogenetic" epilepsy, characterized by brief impaired awareness at spontaneous resolution, coupled to tonic-clonic jerks of the limbs. Drug resistant epilepsy.			
37	<ul style="list-style-type: none"> Unchanged drug resistant epilepsy treated with vigabatrin, oxcarbazepine and phenobarbital. Mild mental retardation (WAIS-R, QI 70). Neurological examination: mild camptocormia with a slight wide-based gait, possible both on toes and on heels, difficult in tandem. Ocular movements assessment was not performed, due to right eye retinal detachment at age 30, with secondary glaucoma in the same eye and severe myopia.	ERT was started (60 Units/kg/every other week)	No effect on critical episodes.	
60	General worsening of the neurological involvement	Ambroxol treatment was started: gradually titrated to a maximum dosage (1300 mg/daily in 3 doses)	Four months after the maximum dose was reached, no effects were observed in the frequency of the critical episodes. Camptocormia and gait remained stable and the EEG was unchanged.	Mild abdominal discomfort at the higher dose
61		Ambroxol treatment was stopped		

additional factors other than the *GBA1* mutation itself might modify the response to Ambroxol. Indeed, it is likely that differences in the complex ER network involved in proteostasis play a key role in this process [16]. Therefore, testing *in vitro* the response to Ambroxol before starting chaperone therapy even in patients carrying responsive *GBA1* mutations, seems mandatory. As expected from *in vitro* results, no clinical effect on epilepsy was observed in P2.

Considering the results reported here and those previously published, it is quite clear that although Ambroxol represent a therapeutic alternative for some nGD patients, additional therapeutic strategies such

as gene therapy and substrate reduction therapy with agents able to cross the blood brain barrier, need to be developed.

In conclusion, our report expands the experience of Ambroxol treatment in nGD patients and highlights the need to *in vitro* test the individual response to Ambroxol even in patients carrying mutations already classified as responding to the chaperone.

Finally, we can translate this concept to all patients with severe GD who present a poor response to other first line therapeutic approaches (i.e. GD with severe bone involvement).

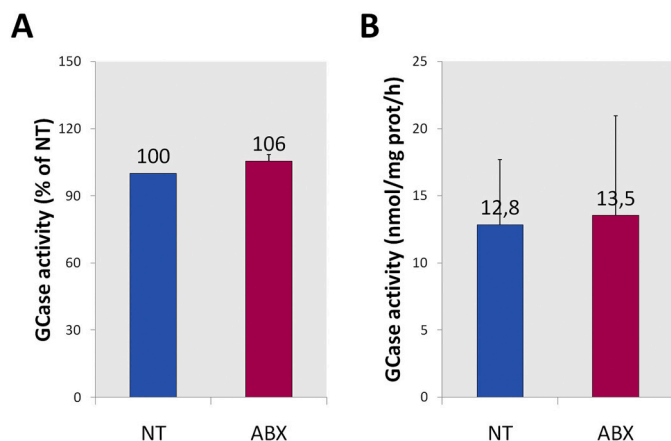


Fig. 3. GCase activity in P2 fibroblasts.

Fibroblasts were treated with Ambroxol hydrochloride 100 μM (ABX) or with an equal volume of vehicle (0,1% DMSO; NT). Values are expressed as the percentages of each NT patient (A) or as nmol/mg protein/h (B). Results are expressed as mean ± SD of three independent experiments, each performed in duplicate.

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