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Pharmacological chaperones increase residual β-galactocerebrosidase activity in fibroblasts from Krabbe patients

Anna Sara Berardi [a,1], Giovanna Pannuzzo [b,1], Adriana Graziano [b], Elvira Costantino-Ceccarini [a], Paola Piomboni [a], Alice Luddi [a,*]

[a] Department of Molecular and Developmental Medicine, University of Siena, Siena, Italy
[b] Department of Bio-Medical Sciences, Section of Physiology, University of Catania, Catania, Italy

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ABSTRACT

Krabbe disease or globoid cell leukodystrophy is a degenerative, lysosomal storage disease resulting from the deficiency of β-galactocerebrosidase activity. This enzyme catalyzes the lysosomal hydrolysis of galactocerebroside and psychosine. Krabbe disease is inherited as an autosomal recessive trait, and many of the 70 disease-causing mutations identified in the *GALC* gene are associated with protein misfolding. Recent studies have shown that enzyme inhibitors can sometimes translocate misfolded polypeptides to their appropriate target organelle bypassing the normal cellular quality control machinery and resulting in enhanced activity. In search for pharmacological chaperones that could rescue the β-galactocerebrosidase activity, we investigated the effect of α-Lobeline or 3′,4′,7-trihydroxyisoflavone on several patient-derived fibroblast cell lines carrying missense mutations, rather than on transduced cell lines. Incubation of these cell lines with α-lobeline or 3′,4′,7-trihydroxyisoflavone leads to an increase of β-galacocerebrosidase activity in p.G553R + p.G553R, in p.E130K + p.N295T and in p.G57S + p.G57S mutant forms over the critical threshold. The low but sustained expression of β-galactocerebrosidase induced by these compounds is a promising result; in fact, it is known that residual enzyme activity of only 15–20% is sufficient for clinical efficacy. The molecular interaction of the two chaperones with β-galactocerebrosidase is also supported by in *silico* analysis. Collectively, our combined *in silico–in vitro* approach indicate α-lobeline and 3′,4′,7-trihydroxyisoflavone as two potential pharmacological chaperones for the treatment or improvement of quality of life in selected Krabbe disease patients.

1. Introduction

Globoid cell leukodystrophy (GLD), also known as Krabbe disease, is a monogenic lysosomal storage disorder (LSD) inherited as an autosomal recessive trait [1,2]. GLD is characterized by a deficiency in galactocerebrosidase (GALC), a lysosomal enzyme essential for normal catabolism of galactolipids, including a major myelin component, galactocerebroside and psychosine [1]. The characteristic biochemical feature of Krabbe disease is the lack of accumulation of the undegraded galactocerebroside in brain, explained by the early degeneration of the myelin forming cells and the block in the synthesis of galactocerebroside [3,4]. However, GALC deficiency results in abnormal accumulation of psychosine, a toxic metabolite which has been demonstrated to induce apoptotic death in oligodendrocytes and Schwann cells throughout respectively the central nervous system (CNS) and peripheral nervous system (PNS) [5–7]. Loss of these myelin-forming cells causes demyelination in both the CNS and PNS during early developmental stages [8,9].

The biochemical disturbances in GLD are manifested by different degrees of brain demyelination, resulting in a broad neurological spectrum of the disease. The rapidly progressive early infantile form is the most common phenotype, which manifests within the first 6 months of life and is characterized by severe neuro-developmental delay, progressive motor dysfunction, and early death [10]. Late onset forms of GLD represent approximately 10% of cases and include the juvenile and adult onsets that predominantly present with motor weakness, sensory and motor neuropathy, cognitive impairment, and psychiatric/behavior disturbances [5,10].

More than 140 mutations have been identified in patients with all clinical types of GLDs, many of which occur in compound heterozygote patterns in patients [2,11–16]. As with many other lysosomal storage diseases, late-onset Krabbe patients with similar or identical genotypes can have varied clinical presentations and course of their disease [13,17]. In fact, while some mutations clearly result in the infantile form if homozygous or heterozygous with another severe mutation, for most of the mutations it is difficult to establish a genotype–phenotype

Abbreviations: GALC, β-galactocerebrosidase; GLD, globoid cell leukodistrophy; ERT, enzyme replacement therapy; CNS, central nervous system; PNS, peripheral nervous system; LSDs, lysosomal storage diseases.

* Corresponding author at: Department of Molecular and Developmental Medicine, Policlinico Le Scotte, viale Bracci, 53100 Siena, Italy.
E-mail address: alice.luddi@unisi.it (A. Luddi).

[1] These authors contributed equally to this work.

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correlation. A more detailed understanding of individual GALC mutations must be established to develop a tailored therapy for Krabbe disease, an approach never taken into consideration for this pathology.

The only available therapy for Krabbe disease is hematopoietic cell transplant using bone marrow or umbilical cord blood cells from healthy donors. This treatment, if done in pre-symptomatic patients, can prevent the rapid neurological course of the disease and the long-term outcome of transplanted infants [18].

Direct intracerebral injection of vectors or transplantation of enzyme-producing cells in the mouse model of the disease has been demonstrated to be effective in enzyme deficiency correction by promoting donor-to-recipient cross correction via enzyme secretion-recapture [19–21]. However, in humans, safety concerns may represent a limit for this approach.

The goal of our study was to assess the therapeutic potential of a pharmacological chaperone therapy approach, a novel and emerging therapeutic strategy using small molecules as drugs for the treatment of Krabbe disease. This therapeutic strategy has the potential to treat diseases caused by missense mutations that result in the synthesis of improperly folded lysosomal enzymes. Indeed, pharmacological chaperones are low-molecular-weight molecules designed to selectively work as a folding template for the conformational mutant enzymes, thereby facilitating proper folding and increasing rescuing of misfolded mutant proteins from the endoplasmic reticulum-associated degradation. Due to their small size, pharmacological chaperones have the potential to be orally available with broad biodistribution, including the CNS.

The use of small pharmaceutical chaperones for the treatment of Krabbe disease is based not only on the safety problems and unsatisfactory results obtained with gene therapy, but it is also suggested by *in vitro* studies done on primary oligodendrocytes demonstrating that over expression of the GALC enzyme is detrimental for oligodendrocytes [19]. It is known that GALC, as other lysosomal enzymes, is constitutively expressed at low levels in all tissues. Our attempt to restore low but sustained expression of the enzyme activity is a promising approach, since it is known that in other lysosomal storage diseases a residual enzyme activity of only 15–20% is sufficient for clinical efficacy [22,23].

In the present study, we screened several pharmacological chaperones and tested them on several fibroblast cell lines from infantile, juvenile and adult Krabbe patients bearing diverse missense mutations. We have identified two potential candidates: α-lobeline already known as a weak inhibitor of GALC [24] and 3′,4′,7-trihydroxyisoflavone a novel chaperone, never previously tested on GALC. The approach used for identification and characterization of the two chaperones was based on biochemical studies in cellular systems and on in *silico* analysis on the interaction between the compounds and the protein. Our studies clearly show that both compounds selectively increase the residual activity in diverse forms of the defective protein. We hope that this approach will be further explored for the development of novel therapeutic treatment of Krabbe patients with responsive mutations, particularly those with severe brain damage.

2. Experimental procedures

2.1. Materials

Fibroblasts from patients with Krabbe disease (p.E130K + p.N295T, p.D187V + p.G323R, p.G286D + p.P318R) were kindly provided by the genetic bio-bank of the Gaslini Institute (Genova, Italy). The fibroblast cell lines carrying the p.G553R + p.G553R and the p.G57S + p.G57S mutation were gift of Prof Balestri (University of Siena) and Prof Federico (University of Siena) respectively. The above GALC mutations are numbered according to HGVS nomenclature recommendations, numbering from the first methionine of the complete 42-residue signal sequence. Since the traditional nomenclature uses p.M17 as the first residue, to switching from new to original nomenclature is required to subtract 16 from their numbers.

The α-lobeline and 3′,4′,7-trihydroxyisoflavone were purchased from Santa Cruz Biotechnology. Stock solutions were prepared in DMSO (Sigma Aldrich) at a concentration of 10 mM, filtered sterile and stored at 20 °C. Fluorogenic substrates were purchased from Moscerdam Substrates or from Sigma Aldrich.

2.2. GALC enzymatic activity assay

Enzyme activity of cerebroside-ß-galactosidase was measured using the fluorogenic substrate 6-hexadecanoylamino-4-methylumbelliferyl-ß-D-galactoside (HMU-ßGal) as originally described [25]. Preliminary studies comparing natural and HMU-ßGal substrate were carried out in order to test the reliability of the water-soluble substrate for this study. No significant differences in the results were obtained; we have therefore decided to use the water-soluble substrate.

Duplicates from each sample, containing 10 μg of total protein from normal control fibroblasts and 20 μl of substrate solution, were mixed on a 200 μl PCR tube and incubated at 37 °C for 17 h. After incubation, 200 μl of stop solution (0.5 M $NaHCO_3$/0.5MNa_2CO_3 buffer pH 10.7 + 0.25% TritonX-100) was added and mixed. The absorbance of the supernatant was measured (excitation at 404 nm and emission at 460 nm) with an F-4500 fluorescence spectrometer (Hitachi, Tokyo, Japan).

2.3. In vitro inhibition assay on GALC

The described GALC enzymatic activity assay was used as the screening method to identify GALC inhibitors. Tested drugs or vehicle DMSO (4% *v*/*v*) were added to the mixture solution containing 10 μg of total protein from normal control fibroblasts and 20 μl of substrate solution. The final concentration of the two compounds in the sample was 0.4 mM and each sample was run in triplicate.

2.4. In vitro inhibition assay for other lysosomal enzymes

Measurements of β-glucosidase, β-galactosidase, α-glucosidase and α-galactosidase enzymatic activity were performed using cultured cell lysates from primary fibroblasts as previously described [26–29].

2.5. Compound toxicity in cell culture

Normal control fibroblast cells were grown in Dulbecco's modified Eagle medium media (Sigma Aldrich) containing 4.5 g/l glucose, 10% fetal calf serum, stable glutamine and 1% antibiotics and maintained at 37 °C in a humidified CO_2 incubator. Cells seeded at 2×10^3 cells/well in a 96-well plate were treated with of α-lobeline or 3′,4′,7-trihydroxyisoflavone at increasing concentrations (10 to 600 μM) for 72 h. To determine the cell toxicity of the two compounds cell viability was assessed using XTT assay kit (Sigma Aldrich). This assay is based on the reduction of tetrazolium salts (XTT) to formazan by the succinate-tetrazolium reductase system in the mitochondria of metabolically active cells. The assay kit was used according to the manufacturer's instructions. The cells were incubated with the tetrazolium salts for 4 h and absorbance was measured at 450-nm using a 550 Ultramark microtiter plate reader (Biorad, Milan, Italy). The experiments were run in triplicates and the results refer to the mean of the three measurements.

2.6. Cell-based chaperone assay for restoring GALC activity

Fibroblast cell lines from patients were grown in Dulbecco's modified Eagle's medium (Sigma Aldrich) and 10% fetal bovine serum (Lonza, Basel, Switzerland) supplemented with 1% penicillin/streptomycin (Lonz,a Basel, Switzerland). Cell lines were cultured at 37 °C in a humidified incubator with 5% CO_2. Prior to treatment, cells were cultured for at

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