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Selective chaperone effect of aminocyclitol derivatives on G202R and other mutant glucocerebrosidases causing Gaucher disease

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ABSTRACT

Gaucher disease is an autosomal recessive lysosomal disorder characterized by the accumulation of glucosylceramide as a result of a deficiency of the enzyme glucocerebrosidase. Several competitive glucocerebrosidase inhibitors are able to act as pharmacological chaperones for an efficient rescue of the mutated, misfolded forms of the enzyme. Along this line, we report in this work on the ability of several aminocyclitols to increase the residual glucocerebrosidase activity in patient fibroblasts with different genotypes. Some of the compounds were slightly active on fibroblasts bearing some mutations, including the highly prevalent N370S mutation. All compounds were highly active as enzyme activity enhancers on fibroblasts from Gaucher disease patients containing the G202R mutation. Moreover, using the novel tagged sphingolipid ω-azidosphingosine, a reduction in the tagged glucosylceramide accumulation was also observed for selected aminocyclitols. Attempts to explain the activity impairment observed in glucocerebrosidase bearing the G202R mutation by comparative molecular dynamic studies on wild type and the G202R mutated proteins (free and isofagomine-bound, in both cases) were unsuccessful. Under the simulation conditions used, no clear effect of the G202R mutation neither over the global structure of the protein nor on the loops that constitute the glucocerebrosidase active site was observed. Since the G202R residue is located on the protein surface, altered protein-membrane or protein-protein interactions could account for the observed differences. In conclusion, we have tested novel compounds that have shown some chaperone effect on particular glucocerebrosidase mutant enzymes, supporting the enhancement therapy as an alternative approach for Gaucher disease.

Abbreviations: BBB, blood–brain barrier; CBE, conduritol β-epoxide; Cer, ceramide; D-MEM, Dulbeco Eagle's minimal essential medium; ERT, enzyme replacement therapy; GBA1, lysosomal glucocerebrosidase; GBA2, non-lysosomal glucocerebrosidase; GlcCer, glucosylceramide; GD, Gaucher disease; GlcCerS, glucosylceramide synthase; IFG, isofagomine; LCS, lactosyl ceramide synthase; MD, molecular dynamics; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NBDNJ, *N*-butyldeoxynojirimycin; NNDNJ, *N*-(*n*-nonyl)deoxynojirimycin; RMSF, root mean square fluctuation; Sap C, saposin C; SM, sphingomyelin; WT, wild type; ωN_3Sph, ω-azidosphingosine.

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1. Introduction

Lysosomal storage diseases are a group of disorders mainly caused by the loss of function of lysosomal enzymes, which leads to the intra-lysosomal storage of non-degraded substrates. The disorders are classified on the basis of the substrate that is accumulated. Gaucher disease (GD, OMIM 230800) is the most prevalent sphingolipidosis caused by the deficiency of glucocerebrosidase (GBA1, E.C. 3.2.1.45), which produces the progressive accumulation of glucosylceramide (GlcCer). Clinically, GD is classified into three major types depending on the absence (type I) or presence (type II and III) of central nervous system involvement. The main symptoms of GD are anemia, thrombocytopenia, hepatosplenomegaly and skeletal disease, as well as neurological features in types II and III (Beutler and Gelbart, 1997; Grabowski, 2008).

Two disease-specific therapies have been approved to treat GD. Enzyme replacement therapy (ERT) has been applied for more than 15 years and has proved successful mainly for visceral and hematological symptoms of type I patients (Grabowski, 2008). However, since the recombinant enzyme does not cross the blood–brain barrier (BBB), its efficacy in neurological involvement is, if any, limited (Schiffmann et al., 1997). Moreover, it is an expensive lifetime treatment that requires frequent intravenous infusion of the enzyme. The other approved treatment is substrate reduction therapy. It is based on the inhibition of glucosylceramide synthase (GlcCerS), the enzyme involved in the rate-limiting first step in the glycosphingolipid biosynthetic pathway, by the oral administration of *N*-butyldeoxynojirimycin (NBDNJ, miglustat, Zavesca®, Fig. 1) (Cox et al., 2000). This reduction therapy is used for type I patients for whom ERT is not a therapeutic option. The small size of NBDNJ makes it of potential use for neurological forms of the disease.

On the other hand, to date other alternative strategies, such as gene therapy, have had very limited success in the treatment of GD. However, new experimental approaches either for conventional gene therapy or based on the partial inhibition of the GlcCerS gene using siRNAs, have been evaluated in cellular and animal models (Enquist et al., 2006; McEachern et al., 2006) (Diaz-Font et al., 2006) with promising results.

In the last few years, a new line of research based on the use of pharmacological chaperones has emerged (Fan, 2003). This approach is based on the assumption that some mutations cause or result in enzyme misfolding and its premature degradation in the endoplasmic reticulum (ER), thereby preventing enzyme transport to the lysosome. In this scenario, a pharmacological chaperone would be able to partially stabilize the mutant protein, thereby allowing its transport to the final destination (Benito et al., 2011; Boyd et al., 2013; Parenti, 2009). A number of iminosugars (Goddard-Borger et al., 2012) and aminocyclitols (Suzuki, 2013) have shown an *in vitro* chaperone-like profile towards GBA1. The first molecule reported to act as pharmacological chaperone *in vivo* for GD was the iminosugar *N*-(*n*-nonyl)deoxynojirimycin (NNDNJ, Fig. 1) (Sawkar et al., 2002). The addition of sub-inhibitory concentrations of NNDNJ to fibroblasts of a GD patient, homozygous for the N370S mutation, resulted in a twofold increase in GBA1 activity (Sawkar et al., 2002). Furthermore, several mutant GBA1 enzymes were shown to increase their activity following the addition of the related iminocyclitol NBDNJ to COS cells transfected with the corresponding mutant cDNAs (Alfonso et al., 2005; Sanchez-Olle et al., 2009). Other iminosugars were also active on the N370S mutant enzyme (Chang et al., 2006; Steet et al., 2006) as well as on several other mutant glucocerebrosidases (Sawkar et al., 2005; Yu et al., 2007). In addition, some non-sugar GBA1 inhibitors (Bendikov-Bar et al., 2013, Marugan et al., 2012) and isofagomine (IFG, Fig. 1) (Lieberman et al., 2007) also showed a chaperone effect on the N370S enzyme in GD patient cells. Unfortunately, although clinical trials using IFG tartrate (AT2101) were initially conducted and showed promising results (Weinreb, 2008), its advancement to phase III has been discontinued.

The knowledge of structural aspects of GBA1 in its native state (Dvir et al., 2003) with different degrees of glycosylation (Brumshtein et al., 2010, Brumshtein et al., 2006, Shaaltiel et al., 2007) under different pH conditions (Lieberman et al., 2009), complexed with different inhibitors (Brumshtein et al., 2009, Brumshtein et al., 2007, Lieberman et al., 2007, Premkumar et al., 2005) and bearing the common N370S mutation (Wei et al., 2011), has opened new avenues in this line of research. Along this line, in a previous work, we undertook a computational study addressed at the prediction of structure-activity relationships for the binding of aminocyclitols generated by our group to GBA1 (Díaz et al., 2011).

As a continuation of our work on the development of new pharmacological chaperones for GD, we here report on the effect of a selection of our recently synthesized aminocyclitols **LD**, (Diaz et al., 2010; Díaz et al., 2011) on the residual GBA1 activity in fibroblasts from patients with diverse genotypes, compared with those of NNDNJ and IFG.

2. Materials and methods

2.1. Chemicals

NNDNJ was purchased from Tocris Bioscience (UK) and IFG (D-tartrate) was purchased from Toronto Research Chemicals, Toronto (Canada). CBE (conduritol β-epoxide) was obtained from a synthetic route described elsewhere (Jaramillo et al., 1994). LD aminocyclitols (Fig. 1) were obtained as previously described (Diaz et al., 2010; Díaz et al., 2011). ω-Azidosphingosine (ωN_3Sph) was synthesized following reported protocols (Garrido, 2012; Garrido et al., 2012). All compounds were dissolved in DMSO and control experiments were performed with DMSO (<1%).

2.2. Fibroblast culture assay

Skin fibroblasts were obtained from non-neurological (type I) and neurological (types II and III) GD patients with distinct genotypes, which were diagnosed, in most of the cases (genotypes: N370S/N370S, L444P/L444P, [D409H;H255Q]/[D409H;H255Q], G202R/G202R) at the Institute of Child Health, Athens. In two cases (N370S/L444P and G202R/[L444P;E326 K]), diagnosis was performed at the Institut de Bioquímica Clínica, Barcelona. Fibroblasts from healthy individuals were used as controls. The study had the approval of the Ethics committees of both institutions. Fibroblast cultures were established following routine procedures in Dulbeco Eagle's minimal essential medium (D-MEM) with 10% FBS.

For the assay of GBA1 in intact cells, 10,000 cells were plated into 24-well plates during 6 days in D-MEM, 10% FBS at 37 °C under 5% CO_2 either with or without NNDNJ, IFG or LD aminocyclitols (see Fig. 1) at several concentrations. Culture media was replaced every 3 days with fresh media supplemented with the corresponding compound dissolved in DMSO. Cells were washed and the enzyme assay performed as follows: substrate (100 μl, 5 mM 4-metylumbelliferyl-β-D-glucoside) in 0.1 M acetate buffer (pH 5.2) was added to each well up to a total volume of 260 μl; incubation was for 1 h at 37 °C. Enzyme reaction was stopped with 2 ml of NaOH-glycine buffer (pH 10.2) and the released 4-methylumbelliferone was measured with a Spectramax M5 plate reader (Molecular Devices), at λex: 340 nm; λem:460 nm. For each experiment untreated (no compound added) and treated cells were plated in quadruplicate. The non-specific GBA1 activity was evaluated by addition of CBE (2 h 0.5 mM) to control wells and was shown to account for about 1% of the activity in control fibroblasts.