EVALUATION OF THE EFFECT OF INHIBITION OF LRRK2 KINASE ACTIVITY ON GLUCOCEREBROSIDASE ACTIVITY ON PATIENT-SPECIFIC CELLS FROM PATIENTS WITH GAUCHER DISEASE

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Abstract. Biallelic mutations in the *GBA1* gene, encoding the lysosomal enzyme glucocerebrosidase (GCase), lead to the development of a lysosomal storage disease, Gaucher disease (GD), and are also a high risk factor for a common neurodegenerative disease, Parkinson's disease (PD). In most cases, mutations in the *GBA1* gene are localized outside the active site and lead to a decrease in GCase activity due to a decrease in the efficiency of transport of the enzyme with an altered conformation into the lysosome. Drugs that are used to treat GD (enzyme replacement therapy) are not able to cross the blood-brain barrier and are not effective for the treatment of neuronal forms of GD or PD associated with mutations in the GBA1 gene (GBA1-PD). For the treatment of PD, drugs that inhibit the kinase activity of leucine-rich repeat kinase 2 (LRRK2) are currently undergoing clinical trials. It was previously shown that inhibition of LRRK2 kinase activity leads to an increase in GCase activity in patient-specific GBA1-PD cells. We first assessed the effect of the kinase activity inhibitor LRRK2 (MLi-2) on GCase activity in a primary culture of peripheral blood macrophages obtained from patients with type 1 GD. Assessment of GCase activity and its substrate levels in cells cultured with and without MLi-2 was performed using high-performance liquid chromatography coupled with tandem mass spectrometry. There was no effect of inhibition of LRRK2 activity on GCase activity in the group of patients with GD.

Keywords: *Gaucher disease, peripheral blood macrophages, GBA1, GCase, LRRK2, inhibitor, enzyme activity*

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INTRODUCTION

Gaucher disease (GD) is a rare autosomal recessive disease belonging to the class of lysosomal accumulation diseases (LADs). The pathogenesis of HD is based on decreased activity of lysosomal glucocerebrosidase hydrolase (GCase) through biallelic mutations in the *GBA1* gene (OMIM 606463) [1]. turn, GCase dysfunction leads to the accumulation of the GCase substrate, the sphingolipid glucocerebroside, predominantly in the lysosomes of liver, spleen and bone marrow cells. There are three main clinical forms of HD. Type 1 is the most common form and is traditionally considered the only type in which patients usually do not have primary neurologic abnormalities. Types 2 and 3 are quite rare, are associated with varying degrees of neurologic impairment, and can be fatal at a young age; in particular, type 2 can manifest perinatally [2, 3]. Depending on the severity of HD course and the severity of GCase activity reduction, mutations in the *GBA1* gene are classified as "mild" (residual GCase activity is 20-35%), e.g., p.N370S, p.V394L, p.R463C, and "severe" (residual GCase activity is 5-10%), e.g., p.L444P, p.R120W, p.Leu29fs (84GG) [4].

Both heterozygous and homozygous carriers of mutations in the *GBA1* gene have an increased risk of developing a common neurodegenerative disease, Parkinson's disease (PD), in different populations [5]. We and other authors have shown that patients with PD associated with mutations in the *GBA1* gene (GBA1-BP) are characterized by decreased GCase activity in blood cells and accumulation of GCase substrate, glucocerebroside, which undergoes deacetylation in the human body and is converted into glucosylsphingosine. However, in GBA1-BP, changes in enzyme activity and substrate concentration are less pronounced than in HD [6-9].

To date, there is no neuroprotective therapy for both HD types 2 and 3 and PD, in particular GBA1-BP. However, the commonality of the pathogenesis of HD and GBA1-BP as diseases associated with GCase dysfunction suggests that targeting drugs aimed at increasing GCase activity may be effective for both GBA1-BP and HD. Peripheral blood macrophage culture has been widely used to study the pathogenesis of diseases associated with GCase dysfunction and to screen potential drugs [10-12].

We and other authors have shown that increased GCase activity in patient-specific cells of GBA1-BP patients can be led by inhibition of kinase activity enriched leucine-rich repeat kinase 2 (LRRK2). LRRK2 is encoded by the *LRRK2* gene, mutations in which lead to an autosomal dominant form of PD due to increased kinase activity, resulting in impaired LRRK2 function in the cell, in particular impaired cellular transport through impaired phosphorylation of LRRK2 substrates, the GTPases of the Rab family [13, 14]. Currently, inhibitors of LRRK2 kinase activity (DNL201, DNL151, ClinicalTrials.gov identifiers NCT04557800, NCT05119790, NCT04056689, NCT05005338, NCT05418673, NCT05348785, NCT 05229562, NCT04551534, NCT03710707 and NEU-723, ClinicalTrials.gov identifiers.gov NCT05633745; note: BIIB122 is an alternative name for DNL151) and one antisense oligonucleotide (BIIB094, ClinicalTrials.gov identifier NCT03976349) are undergoing clinical trials for PD therapy [15].

The effect of LRRK2 kinase activity inhibitors on GCase activity was first demonstrated by Ysselstein et al. [16] on dopaminergic neurons (DN) differentiated from induced pluripotent stem cells and confirmed in further studies [16-19]. In turn, we have shown an increase in GCase activity upon LRRK2 inhibition on primary culture of peripheral blood macrophages from GBA1-BP patients [17]. It has been suggested that the increase in GCase activity upon inhibition of LRRK2 kinase activity may result from decreased phosphorylation of Rab10 protein, which is involved in endolysosomal transport, resulting in increased GCase transport into lysosomes [16, 17, 19, 20]. Whether LRRK2 inhibitors can increase activity in the presence of biallelic mutations, particularly in HD, remains unknown.

The aim of this study was to evaluate the effect of inhibitor of LRRK2 kinase activity (MLi-2) on GCase activity and hexazylsphingosine (HexSph) concentration in primary culture of macrophages from PD patients. GBA1-BP patients and a group of neurologically healthy individuals were also included in the study as comparison groups.

MATERIALS AND METHODS

Characterization of the groups included in the study. The study included 7 patients with HD, 9 patients with GBA1-BP, and 8 neurologically healthy individuals (controls). The clinical demographic characteristics of the study groups are summarized in Table 1. Patients with HD are observed at the V.A. Almazov National Medical Research Center. All patients with HD are treated with enzyme replacement therapy. Blood sampling was performed before the introduction of this therapy.

Table 1. Clinical and demographic characteristics of the study groups

groups	BG	GBA1-BP	Control
Numbers (*n*)	7	9	8
Gender (male/female)	4/3	1/8	2/6
Age, years	30,00 (21,00-56,00)	62,50 (48,00-73,00)	62,00 (54,00-72,00)
Mutations in the *GBA1* gene	2 - p.N370S/p.L444P 1 - p.N370S/p.Leu29fs 2 - p.N370S/p.R120W 1 - p.N370S/p.N370S 1 - p.N370S/NA	5 - p.N370S/N 4 - p.L444P/N	-
GCase activity in blood, mmol/liter/hr	0,32 (0,01-0,96) p.N370S/p.L444P: 0.43 (0.01-0.51) p.N370S/p.Leu29fs: 0.06 (0.02-0.09) p.N370S/p.R120W: 0.48 (0.18-0.82) p.N370S/p.N370S: 0.96 (0.96-0.96) p.N370S/NA: 0.64 (0.64-0.64)	3,2 (1,42-5,07) p.N370S/N: 3.77 (2.32-5.07) p.L444P/N: 2.78 (1.42-4.96)	4,69 (2,85-12,87)
HexSph concentration in blood, ng/mL	223,59 (44,82-737,17) p.N370S/p.L444P: 279.67 (77.7-381.73) p.N370S/p.Leu29fs: 68.14 (47.29-122.28) p.N370S/p.R120W: 597.22 (347.99-737.17) p.N370S/p.N370S: 196.16 (196.16-196.16) p.N370S/NA: 44.82 (44.82-44.82)	4,38 (1,7-6,19) p.N370S/N: 4.15 (1.7-6.19) p.L444P/N: 4.62 (2.36-5.67)	5,42 (1,61-14,47)
GCase activity in primary culture of	0,16 (0,01-2,58) p.N370S/p.L444P: 0.43	6,26 (1,58-15,34) p.N370S/N: 13.04	15,94 (2,86-48,24)

peripheral blood macrophages, mmol/liter/hr	(0.01-0.72) p.N370S/p.Leu29fs: 0.01 (0.01-0.09) p.N370S/p.R120W: 0.45 (0.16-0.84) p.N370S/p.N370S: 2.58 (2.58-2.58) p.N370S/NA: 0.01 (0.01-0.01)	(8.96-15.34) p.L444P/N: 3.46 (1.58-11.47)	
Concentration of HexSph in primary culture of peripheral blood macrophages, ng/mL	3,17 (0,01-20,19) p.N370S/p.L444P: 2,95 (2,55-3,32) p.N370S/p.Leu29fs: 16.27 (4.18-20.19) p.N370S/p.R120W: 2.14 (0.01-10.35) p.N370S/p.N370S: 0.99 (0.99-0.99) p.N370S/NA: 1.79 (1.79-1.79)	0,77 (0,08-2,68) p.N370S/N: 1.78 (0.08-2.68) p.L444P/N: 0.68 (0.12-1.96)	0,28 (0,07-1,06)

Note. HexSp, hexazylsphingosine; GCase, glucocerebrosidase; NA, no information; /N, normal (wild-type) allele without mutation.

The diagnosis of PD was made, according to clinical guidelines (https://cr.minzdrav.gov.ru/recomend/24_2), on the basis of symptoms and biochemical evaluation of GCase activity with subsequent detection of mutations in the *GBA1* gene by Sanger direct sequencing. Biochemical analysis and sequencing of the *GBA1* gene in patients with PD was performed in the N.P. Bochkov Medical and Genetic Research Center, Moscow. The diagnosis of PD for one of all patients was confirmed only on the basis of biochemical parameters, in particular GCase activity and HexSph concentration, because sequencing of the *GBA1* gene revealed one mutation in the heterozygous state.

Patients with GBA1-BP are observed at the N.P. Bekhtereva Human Brain Institute of the Russian Academy of Sciences. The diagnosis of PD was established according to previously published criteria [21]. We previously identified patients with GBA1-BP by screening two major

mutations in the *GBA1* gene (p.N370S [NM_000157.4:c.1226A> G], p.L444P [NM_000157.4:c.1448T> C]) among PD patients according to a previously described protocol [5]. This group of patients is described in our study to evaluate the effect of LRRK2 inhibitors on biochemical characteristics in PD associated with mutations in the *GBA1* and *LRRK2* genes on patient-specific cells [17]. The control was recruited from volunteers who are observed in the consultative and diagnostic center of the First St. Petersburg Pavlov State Medical University. All individuals in the control group were also screened for two major mutations in the *GBA1* gene (p.N370S, p.L444P) in order to exclude carriers of these mutations. The groups included in the study differed statistically significantly by sex ($p = 0.03$) and age ($p= 0.0002$).

All study participants signed informed consent. Blood was collected from each study participant for further obtaining primary culture of peripheral blood macrophages.

Cultivation of primary culture of peripheral blood macrophages. Primary culture of peripheral blood macrophages was obtained according to the protocol described previously from the mononuclear fraction isolated from whole blood of each individual [22]. On the fourth day, a selective inhibitor of LRRK2 kinase activity, MLi-2 ("Abcam", USA), was added to the primary macrophage culture at a concentration of 100 nM, followed by culturing for 4 days as previously described by us [17]. The efficiency of MLi-2 inhibitor action was confirmed by the ratio of the phosphorylated form at position Thr73 to the unphosphorylated form of Rab10 protein, which is the main substrate of LRRK2, using Western blotting, as described by us earlier [17].

Evaluation of GCase activity and HexSph concentration. The enzymatic activity of GCase and the concentration of hexazylsphingosine, which is a mixture of two epimers of glycosylsphingosine (GlcSph) and galactosylsphingosine (GalSph), was performed by high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS) on primary culture of peripheral control macrophages in the presence of MLi-2 at a concentration of 100 nM and without it according to the protocol described earlier in the laboratory of hereditary metabolic diseases of the N. P. Bochkov Medico-Genetic Research Center, Moscow.P. Bochkov, Moscow [6, 9]. All measurements were performed in three repetitions.

Statistics. Statistical processing of the obtained data was performed in the R programming environment (version 4.1.2) using built-in statistical packages. The normality of data distribution was checked using the Shapiro-Wilk test. The Wilcoxon test was used to analyze differences between groups. In cases of dependent (paired) samples, the paired Wilcoxon test was used. For independent samples, differences were evaluated using the Wilcoxon test supplemented by post-hoc analysis with Bonferroni correction for multiple comparisons. Values of $p< 0.05$ were considered statistically significant. Clinical characteristics are presented as mean± standard deviation, experimental values as median (minimum/maximum).

RESULTS AND DISCUSSION

Currently, there are no approaches for the treatment of neuronal forms of HD, as well as for the treatment of GBA1-BP. Previously, an increase in GCase activity with inhibition of LRRK2 kinase activity was shown in DN patients with GBA1-BP [17, 19]. We have confirmed the previously obtained data, and for the first time we have shown the effect of LRRK2 activity inhibition on the restoration of GCase activity on the primary culture of macrophages from GBA1-BP patients.

In the present study, we evaluated for the first time the effect of a selective inhibitor of LRRK2 kinase activity on GCase activity and HexSph concentration in the primary culture of peripheral blood macrophages from HD patients. Primary culture of peripheral blood macrophages has been widely used both to study the pathogenesis of HD and to screen potential drugs aimed at increasing GCase activity [10-12]. In the present study, just as we have previously shown, GCase activity was decreased and HexSph concentration was increased in the primary culture of peripheral blood macrophages from HD patients compared to GBA1-BP patients and controls (GCase: p= 0.000043, p= 0.0000074; HexSph: p= 0.00042, p= 0.00012, respectively) (Fig. 1, *a* and *d*). At the same time, GBA1-BP patients with heterozygous carriage of mutations in the *GBA1* gene were characterized by decreased GCase activity (p= 0.013) and increased HexSph concentration (p= 0.0026) in the primary culture of peripheral blood macrophages compared to controls, but these changes were less pronounced (Fig. 1, *a* and *d*) [10, 11].

Fig. 1. Level of GCase and HexSph activity in primary culture of peripheral blood macrophages of patients with HD, GBA1-BP and controls. *a* - GCase activity in primary culture of peripheral blood macrophages of the studied groups; *b* - GCase activity in primary culture of peripheral blood macrophages of the studied groups when cultured in the presence and without LRRK2 kinase activity inhibitor; *c* - GCase activity in primary culture of peripheral blood macrophages of patients with GBA1-BP and HD when cultured in the presence of LRRK2 kinase activity and controls without LRRK2 kinase activity inhibitor; *d* - HexSph concentration in the primary culture of peripheral blood macrophages of the studied groups; *e* - HexSph concentration in the primary culture of peripheral blood macrophages of the studied groups when cultured in the presence and without LRRK2 kinase activity inhibitor; *e* - HexSph concentration in primary culture of peripheral blood macrophages of GBA1-BP and HD patients when cultured in the presence of

LRRK2 kinase activity and controls without LRRK2 kinase activity inhibitor. * $p< 0.05$; ** $p< 0.01$; *** $p< 0.001$; **** $p< 0.0001$; ns - no statistical significance

As we have shown previously, inhibition of LRRK2 kinase activity resulted in a statistically significant entrainment of GCase activity in the primary culture of peripheral blood macrophages in the group of patients with GBA1-BP compared to controls ($p= 0.012$; Fig. 1, *b*),which is consistent with previous findings in DN patients with GBA1-BP [16, 18, 19]. It has been suggested that alteration of LRRK2 kinase activity may affect intracellular transport, particularly of lysosomal enzymes from the endoplasmic reticulum to the lysosome, by altering the phosphorylation level of Rab10 protein, which is the main substrate of LRRK2, which in turn leads to an increase in GCase activity and level [17].

However, no changes in the level of GCase activity upon LRRK2 inhibition were detected in the group of patients with HD and controls ($p> 0.05$; Fig. 1, *b*). Significantly, when LRRK2 activity was inhibited, GCase activity in the group of GBA1-BP patients reached the values of GCase activity in the primary culture of peripheral blood macrophages of controls without MLi-2 inhibitor ($p> 0.05$; Fig. 1, *c*). It is interesting to note that the group of patients with GBA1-BP also showed a decrease in the concentration of HexSph in the primary culture of peripheral blood macrophages when LRRK2 kinase activity was inhibited by MLi-2 inhibitor, while the controls showed a statistically significant decrease in the concentration of HexSph when LRRK2 kinase activity was inhibited ($p= 0.041$; Fig. 1, *e* and *f*). In the group of patients with HD no changes in HexSph concentration upon LRRK2 inhibition were detected ($p> 0.05$; Fig. 1, *e* and *f*).

The primary culture of peripheral blood macrophages of GBA1-BP patients was characterized by a decrease in GCase activity and an increase in HexSph concentration regardless of mutations in the *GBA1* gene (Fig. 2 *a* and *d*). It is interesting to note that the p.L444P mutation does not affect GCase activity but decreases GCase stability, which leads to a decrease in the level of this protein in the cell [23-25]. At the same time, no pronounced effect on GCase protein stability was shown for the p.N370S mutation [26]. It is assumed that in the case of the p.N370S mutation, the total decrease in GCase activity is explained by changes in the affinity of the enzyme for membranes [24]. It is conceivable that the enhancement of GCase transport into the lysosome upon inhibition of LRRK2 activity may depend on the type of mutation. Exposure to an inhibitor of LRRK2 kinase activity resulted in an increase in GCase activity in the primary culture of macrophages from GBA1-BP patients with the p.L444P mutation only ($p= 0.0078$) and reached the level of GCase activity in untreated control cells, but not in the group of GBA1-BP patients

with the p.N370S mutation (Fig. 2, *b* and *c*). However, no effect of LRRK2 inhibitor on HexSph levels was detected (Fig. 2, *e* and *f*). In the present study, all HD patients were compound heterozygotes of the "mild" (p.N370S) and one of the "severe" (p.L444P, p.Leu29fs, p.R120W) mutations.

Fig. 2. Level of GCase and HexSph activity in primary culture of peripheral blood macrophages of patients with HD, GBA1-BP and control depending on mutations in the *GBA1* gene in the group of patients with GBA1-BP. *a* - GCase activity in primary culture of peripheral blood macrophages of the studied groups; *b* - GCase activity in primary culture of peripheral blood macrophages of the studied groups when cultured in the presence and without LRRK2 kinase activity inhibitor; *c* - GCase activity in primary culture of peripheral blood macrophages of GBA1-BP and BG patients when cultured in the presence of LRRK2 kinase activity and controls without LRRK2 kinase activity inhibitor; *d* - HexSph concentration in the primary culture of peripheral blood macrophages of the studied groups; *e* - HexSph concentration in the primary culture of peripheral blood macrophages of the studied groups when cultured in the presence and without LRRK2 kinase activity inhibitor; *e* - HexSph concentration in primary culture of peripheral blood macrophages of GBA1-BP and HD patients when cultured in the presence of LRRK2 kinase activity and controls without LRRK2 kinase activity inhibitor. * $p< 0.05$; ** $p< 0.01$; *** $p< 0.001$; **** $p< 0.0001$; ns - no statistical significance

On the other hand, it cannot be excluded that one functional copy of the *GBA1* gene is sufficient to increase GCase activity by enhancing the transport of functionally active GCase into the lysosome. The absence of changes in the level of GCase activity in the group of patients with HD can be justified by the absence of functional copies of the *GBA1* gene due to biallelic mutations in the *GBA1* gene. Enhancement of GCase enzyme transport into lysosome by inhibition of LRRK2 activity in the presence of only mutant forms of the enzyme is insufficient to increase GCase activity. It should also be taken into account that the absence of the effect of LRRK2 inhibition while increasing GCase activity on the level of HexSph in the primary culture of macrophages of patients with HD may be due to insufficient time of cell cultivation in the presence of MLi-2 or insufficient concentration of the inhibitor.

As a promising strategy for the therapy of GBA1-BP and HD, small chemical compounds called pharmacological chaperones (PS), which are able to penetrate the blood-brain barrier, selectively bind to the GCase enzyme, stabilize its structure, and promote its translocation into the lysosome, which together allows to restore the biological function of this enzyme, have been

developed to date [10, 11]. On patient-specific cells, we and other authors have shown the effectiveness of this class of drugs in restoring GCase activity in HD. Currently, FS are undergoing phase 2 clinical trials for the therapy of HD (NCT03950050) and phase 3 clinical trials for the therapy of PD (NCT05778617). However, GCase FSs have not yet been introduced into clinical practice.

CONCLUSION

Given our data, LRRK2 inhibitors should not be considered as a monotherapy for HD, but it seems important to evaluate the combined effect of LRRK2 inhibitors with drugs that promote the correction of the assembly of the mutant enzyme, such as FSh GCase. Such an approach can simultaneously enhance the enzyme transport into the lysosome and restore the active conformation of GSaSe.

AUTHORS' CONTRIBUTION

T.S. Usenko - concept and guidance of the work; K.S. Basharova, A.I. Bezrukova, G.V. Baidakova, V.A. Bezrukikh - conducting experiments; K.S. Basharova, E.Yu. Zakharova, S.N. Pchelina, T.S. Usenko - discussion of research results; K.S. Basharova statistical processing of data; K.S. Basharova - graphical representation of data; T.S. Usenko - writing the text; T.S. Usenko, S.N. Pchelina, K.S. Basharova - editing the text of the article.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

ETHICS DECLARATION

All procedures performed in research involving human participants conformed to the ethical standards of the national research ethics committee and the 1964 Declaration of Helsinki and its subsequent revisions or comparable ethical standards. Informed voluntary consent was obtained from each of the participants included in the study. The study was approved by the Ethical Committee of the Pavlov First St. Petersburg State Medical University (protocol№ 275 from 04.09.2023).

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