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Dose-Dependent Alterations of Lysosomal Activity and Alpha-Synuclein in Peripheral Blood Monocyte-Derived Macrophages and SH-SY5Y Neuroblastoma Cell Line by upon Inhibition of MTOR Protein Kinase – Assessment of the Prospects of Parkinson's Disease Therapy

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Abstract—To date, the molecular mechanisms of the common neurodegenerative disorder Parkinson's disease (PD) are unknown and, as a result, there is no neuroprotective therapy that may stop or slow down the process of neuronal cell death. The aim of the current study was to evaluate the prospects of using the mTOR molecule as a potential target for PD therapy due to the dose-dependent effect of mTOR kinase activity inhibition on cellular parameters associated with, PD pathogenesis. The study used peripheral blood monocyte-derived macrophages and SH-SY5Y neuroblastoma cell line. As a result, we have for the first time showed that inhibition of mTOR by Torin1 only at a concentration of 100 nM affects the level of the lysosomal enzyme glucocerebrosidase (GCase), encoded by the *GBA1* gene. Mutations in GBA1 are considered a high-risk factor for PD development. This concentration led a decrease in pathological phosphorylated alpha-synuclein (Ser129), an increase in its stable tetrameric form with no changes in the lysosomal enzyme activities and concentrations of lysosphingolipids. Our findings suggest that inhibition of the mTOR protein kinase could be a promising approach for developing therapies for PD, particularly for GBA1-associated PD.

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Keywords: Parkinson's disease, mTOR, Torin 1, alpha-synuclein, glucocerebrosidase, autophagy, lysosomal enzyme activity, lysosphingolipids

INTRODUCTION

Parkinson's disease (PD) is one of the most common neurodegenerative diseases characterized by dopaminergic neuron death in the substantia nigra as well as alpha-synuclein accumulation and aggregation [1]. Precise molecular mechanisms underlying PD development remain unknown. Consequently, there are no neuroprotective drugs capable of reversing or slowing neurodegeneration. Recent studies suggest that the processes such as neuroinflammation, mitochondrial dysfunction, disrupted lipid homeostasis,

Abbreviations: ASMase, acid sphingomyelinase; GBA1-PD, PD associated with mutations in the *GBA1* gene; GCase, glucocerebrosidase; GLA, alpha-galactosidase; HexSph, hexosylsphingosine; LysoGb3, lyso-globotriaosylsphingosine; LysoSM, lysosphingomyelin; PD, Parkinson's disease; sPD, sporadic PD.

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endoplasmic reticulum stress, and impaired autophagolysosomal system may be involved in PD molecular mechanisms [2, 3]. However, altered autophagy, which accounts for about half of cellular alpha-synuclein degradation, is currently recognized as a key factor underlying PD pathogenesis [4-6]. Thus, one of the promising approaches for PD therapy may rely on autophagy regulation primarily via the PI3K/AKT/mTOR pathway [7, 8]. Previously, we and others revealed that both sporadic PD (sPD) and PD caused by the *GBA1* gene mutation (GBA1-PD), one of the most common forms of PD, are associated with impaired PI3K/AKT/mTOR signaling [9-14]. In this regard, transcriptome analysis of GBA1-PD patient-specific cells and a mouse model of parkinsonism triggered by lysosomal glucocerebrosidase (GCase) dysfunction allowed us to identify altered expression of genes regulated by the PI3K/AKT/mTOR cascade [9, 10]. Mutations in the GCase-encoding *GBA1* gene represent a high genetic risk factor for PD development resulting in downregulated GCase activity and level in both homozygous and heterozygous carriers [15, 16]. In turn, when mTOR inhibitors targeted the PI3K/AKT/mTOR pathway, improved alpha-synuclein clearance was observed in both sPD and GBA1-PD cells and mouse models [12, 17, 18]. However, it should be noted that both mTOR hyperactivation and hypoactivation can result in lysosome dysfunction, leading to cell death [19]. Hence, it is crucial to maintain a balance between mTOR signaling activation and lysosomal function. This study aimed to assess to assess a dose-dependent effect of Torin 1-mediated mTOR inhibition on cell parameters associated with PD, primarily activity of lysosomal enzymes and lysosphingolipid levels, autophagy level, contents of alpha-synuclein protein and GCase. The primary peripheral blood macrophages derived from neurologically healthy individuals and the SH-SY5Y neuroblastoma cell line were used in this study because these cell types are widely utilized by us and other researchers for screening potential new drugs for neurodegenerative diseases and for investigating disease-related molecular mechanisms, particularly those associated with GBA1-PD [20-22]. Torin 1 was chosen as an inhibitor of mTOR kinase activity. It has been previously proven to effectively downregulate the level of phosphorylated alpha-synuclein protein (Ser129) and restore the functioning of the autophagolysosomal system in the patient-specific cells derived from the biallelic *GBA1* gene variant carriers (Gaucher disease) as well as patients with GBA1-PD [12, 23].

MATERIALS AND METHODS

Characteristics of study participants. Six neurologically healthy individuals (2 males, 4 females, mean age – 30.3 ± 5.9 years) observed at the consultative and diagnostic center of the Pavlov First Saint Petersburg State Medical University were enrolled in the study.

Primary peripheral blood macrophage culture. Previously, we described the protocol used to obtain a primary peripheral blood macrophage culture followed by the isolation of a whole blood mononuclear fraction from each participant [21, 24, 25]. On day 4 of cultivation, a selective mTOR protein kinase inhibitor Torin 1 (Abcam, USA) was added to the primary macrophage culture at various concentrations (25, 50, 100, 200 nM) which were chosen based on pre-assessed levels of cell survival. The cultures were then incubated for 24 h.

SH-SY5Y neuroblastoma cell line culture. An SH-SY5Y neuroblastoma cell line, courtesy of Dr. Sci. Biol. E. V. Kaznacheeva, Institute of Cytology of the Russian Academy of Sciences, St. Petersburg, was cultured in DMEM medium (Biolot, Russia) supplemented with 10% fetal bovine serum (Biolot) and 1% gentamicin (Biolot), for 4 days at 37°C, 5% CO_2. SH-SY5Y neuroblastoma cell line used in the study underwent not more than seven passages and was differentiated according to a previously described protocol [26]. On day 9 of cultivation a selective mTOR protein kinase inhibitor Torin 1 was added to SH-SY5Y neuroblastoma cell line at various concentrations (25, 50, 100, 200 nM) chose based on the pre-assessed cell survival levels. The cells were then incubated for 24 h. Each experiment was carried out in triplicate.

Survival of the mTOR protein kinase inhibitor-exposed primary peripheral blood macrophage culture and SH-SY5Y neuroblastoma cell line. The macrophages were maintained for 5 days, while the SH-SY5Y cells were cultured for 10 days, following the protocol described earlier. Subsequently, both cell types were exposed to Torin 1 at various concentrations (25, 50, 100, 150, 200, 250, 300, 400 nM) and cultivated for an additional 24 h under similar conditions. Cell survival assessment was performed as previously described [27]. For each Torin 1 concentration, experiments were performed in triplicate.

Immunofluorescence-assessed autophagy level. A primary peripheral blood macrophage culture and SH-SY5Y neuroblastoma cell line treated with Torin 1 at various concentrations based on the pre-assessed cell survival level left untreated, were incubated with LysoTracker-Red DND-99 (Thermo Scientific, USA) for 30 min. Next, the cells were fixed using 4% paraformaldehyde (Sigma-Aldrich, USA) for 30 min, washed with phosphate-buffered saline (Rosmedbio, Russia) and incubated in a 1% bovine serum albumin (Biolot) solution for 30 minutes. After that, the cells were stained with primary anti-LC3B antibodies (ABclonal, USA; A19665; 1 : 500) for 60 min followed by staining

with fluorescent Alexa Fluor 488-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, USA; 1 : 400) for 60 min. Finally, cells were analyzed using a Leica TCS-SP5 confocal microscope (Leica Microsystem GmbH, Germany) and Fiji software (version 2.14.0/1.54f).

Protein levels of phosphorylated mTOR, GCase, alpha-synuclein, and LC3B assessed by Western blotting. Total protein level was quantified by using a Pierce BCA Protein Assay kit (Thermo Scientific, Lithuania). Equal protein amounts were separated by electrophoresis in a polyacrylamide gel (20% SDS-PAGE – for LC3B protein, 12% SDS-PAGE – for other proteins) and transferred to polyvinylidene fluoride membrane (Bio-Rad, USA). Protein level of phosphorylated mTOR (Ser2448), GCase, alpha-synuclein (phosphorylated (Ser129), monomeric and tetrameric forms), LC3B in primary peripheral blood macrophages derived from neurologically healthy individuals and SH-SY5Y neuroblastoma cell line treated with or without an mTOR protein kinase inhibitor at different concentrations were quantified using specific primary antibodies (1 : 1000): Phospho-mTOR-S2448, ABclonal, cat. # AP0094, USA; Glucosylceramidase beta (GBA), ABclonal, cat. # A8420; Phospho-α-Synuclein (Ser129) (D1R1R), Cell Signal, cat. # 23706, USA; anti-alpha-synuclein oligomeric, Sigma, cat. # ABN2265, USA; LC3B, ABclonal, cat. # A19665 and secondary peroxidase-conjugated antibodies (goat anti-rabbit HRP conjugate, Abcam, cat. # ab6721, UK; 1 : 5000). After that, secondary antibodies were added and the formed complexes were quantified using a Clarity Western ECL Blotting Substrate detection system (Bio-Rad). Obtained protein quantities were normalized to GAPDH reference protein (ABclonal, cat. # AC036; 1 : 15,000). For each protein, experiments were performed in triplicate. Western blot data were analyzed using Fiji software (version 2.14.0/1.54f).

Lysosomal enzyme activity and lysosphingolipid concentrations. Enzymatic activity of lysosomal enzymes (GCase, alpha-galactosidase (GLA), and sphingomyelinase (ASMase)) as well as concentrations of the relevant substrates (hexasylsphingosine (HexSph) – a mixture of glycosylsphingosine and galactosylsphingosine; lysosphingomyelin (LysoSM), and lysoglobotriaosylsphingosine (LysoGb3)) were assessed using high-performance liquid chromatography with tandem mass spectrometry in a primary peripheral blood macrophage culture derived from the neurologically healthy individuals and SH-SY5Y neuroblastoma cell line treated with Torin 1 at various concentrations or untreated, following protocols previously described [24, 28-30]. All experiments were performed in triplicate.

Statistical analysis. Statistical data processing was conducted using pre-installed R packages (version 4.3.2) (https://cran.r-project.org/bin/windows/base/). The normality of distribution in the obtained data was assessed using the Shapiro–Wilk test. Inter-group differences were evaluated using the paired Wilcoxon test. A significance level of $p < 0.05$ was considered statistically significant. Clinical characteristics of the study participants are presented as mean ± standard deviation of the mean, experimental values – as median (min-max).

RESULTS

Currently, therapeutic targets for PD treatment are being extensively searched for worldwide. Here, we investigated the dose-dependent effect of mTOR inhibitor on cell parameters known to be associated with PD pathogenesis.

The degree of mTOR inhibition by Torin 1 was evaluated in primary peripheral blood macrophage cultures derived from neurologically healthy individuals and in the SH-SY5Y neuroblastoma cell line. Cell survival was evaluated in both the primary peripheral blood macrophage culture and in the SH-SY5Y neuroblastoma cell line following treatment with the mTOR protein kinase inhibitor Torin 1, aiming to determine its effective concentration range. Based on these assessments, Torin 1 concentrations (25, 50, 100, 200 nM) that reduced cell viability by no more than 80% in both cell models were selected for subsequent experiments.

The dose-dependent inhibition of mTOR by Torin 1 was assessed by measuring the decrease in relative levels of phosphorylated mTOR protein (Ser2448) in the primary peripheral blood macrophage culture derived from neurologically healthy individuals and in the SH-SY5Y neuroblastoma cell line was assessed by analyzing decline in the relative level of phosphorylated mTOR protein (Ser2448) (Fig. 1, a and b). In the primary macrophage culture, Torin 1 showed no significant decrease in phosphorylated mTOR protein levels at any of the tested concentrations compared to untreated cells (Fig. 1c). Conversely, in the SH-SY5Y neuroblastoma cell line, Torin 1 significantly reduced phosphorylated mTOR protein levels at doses of 100 and 200 nM compared to the control ($p < 0.01$ and $p < 0.001$, respectively; Fig. 1d).

Torin 1-mediated dose-dependent protein kinase mTOR inhibition affects autophagy, lysosomal activity, and GCase protein level in the primary peripheral blood macrophage culture derived from the neurologically healthy individuals and in SH-SY5Y neuroblastoma cell line. The level of autophagy in the primary peripheral blood macrophage culture derived from neurologically healthy individuals and in SH-SY5Y neuroblastoma cell line was assessed by analyzing relative level of the hallmark autophagy marker LC3B-II protein along with fluorescent

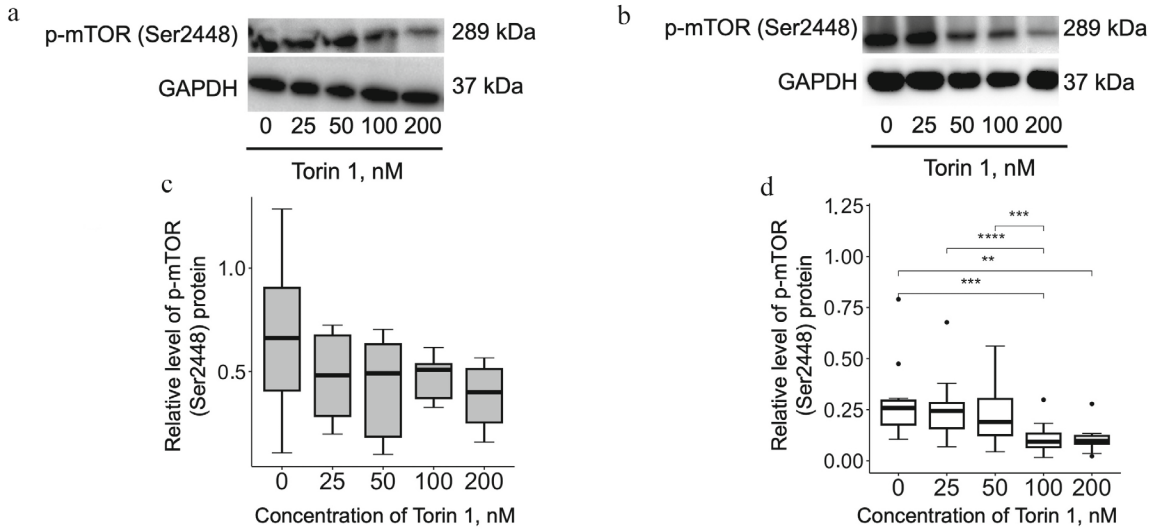

Fig. 1. Dose-dependent Torin 1-mediated effect of mTOR inhibition on the protein level of phosphorylated mTOR (Ser2448). a) Western blot analysis for mTOR phosphorylated (Ser2448) protein level in primary peripheral blood macrophages; b) Western blot analysis for mTOR phosphorylated (Ser2448) protein level in tSH-SY5Y neuroblastoma cell line. c) Relative level of mTOR phosphorylated (Ser2448) protein in primary peripheral blood macrophages (n = 6, where n is the number of independent samples); d) relative level of mTOR phosphorylated (Ser2448) protein level in SH-SY5Y neuroblastoma cell line (n = 5, where n is the number of independent cell line samples). ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

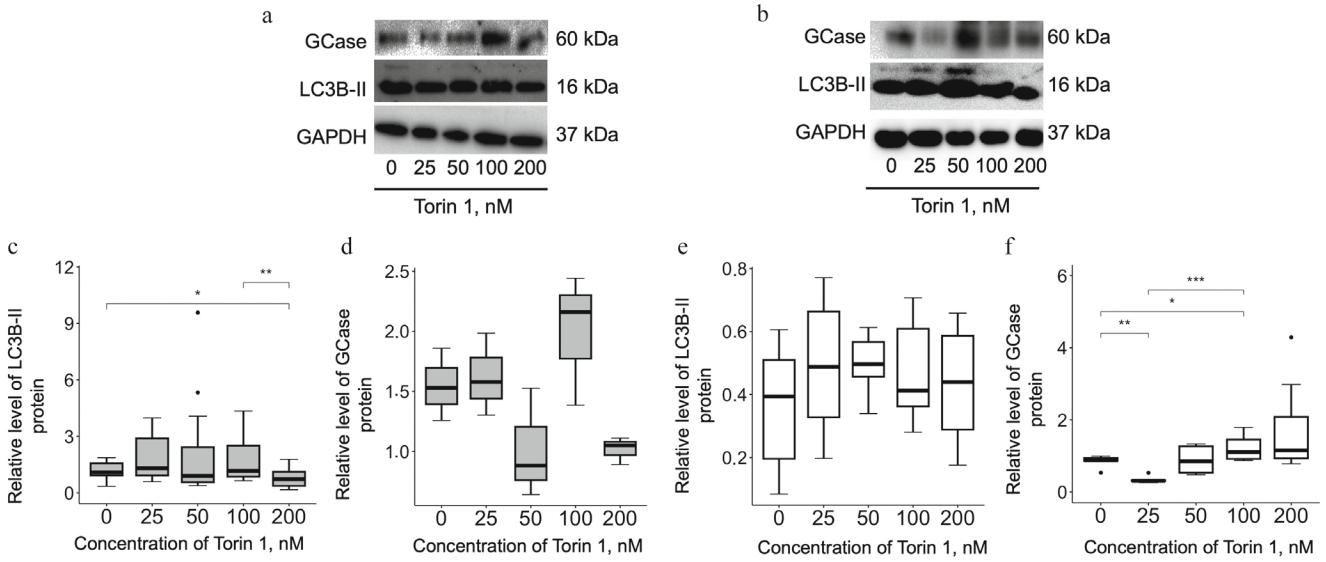

Fig. 2. Torin 1-mediated effect of dose-dependent mTOR inhibition on the levels of LC3B-II and GCase proteins. a) Western blot analysis for LC3B-II and GCase proteins in primary peripheral blood macrophages; b) Western blot analysis for LC3B-II and GCase proteins in SH-SY5Y neuroblastoma cell line. c) Relative level of LC3B-II protein in primary peripheral blood macrophages (n = 6, where n is the number of independent samples); d) relative level of GCase protein in primary peripheral blood macrophages (n = 6, where n is the number of independent samples); e) relative level of LC3B-II protein in SH-SY5Y neuroblastoma cell line (n = 5, where n is the number of independent cell lines); f) relative level of GCase protein in SH-SY5Y neuroblastoma cell line (n = 5, where n is the number of independent cell line samples). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.01$.

staining of LC3B protein and lysosome assuming that colocalization of LC3B with lysosomes could indicate autophagosome-lysosome fusion (Fig. 2, a and b; Fig. 3, a and b) [31].

In primary peripheral blood macrophages derived from neurologically healthy individuals, treatment with Torin 1 at a concentration of 200 nM resulted in a significantly lower relative level of LC3B-II protein compared to cultures exposed to 100 nM Torin 1 and untreated controls ($p < 0.05$; Fig. 2c). Conversely, exposure to lower doses of Torin 1 (25, 50, and 100 nM) tended to insignificantly elevate

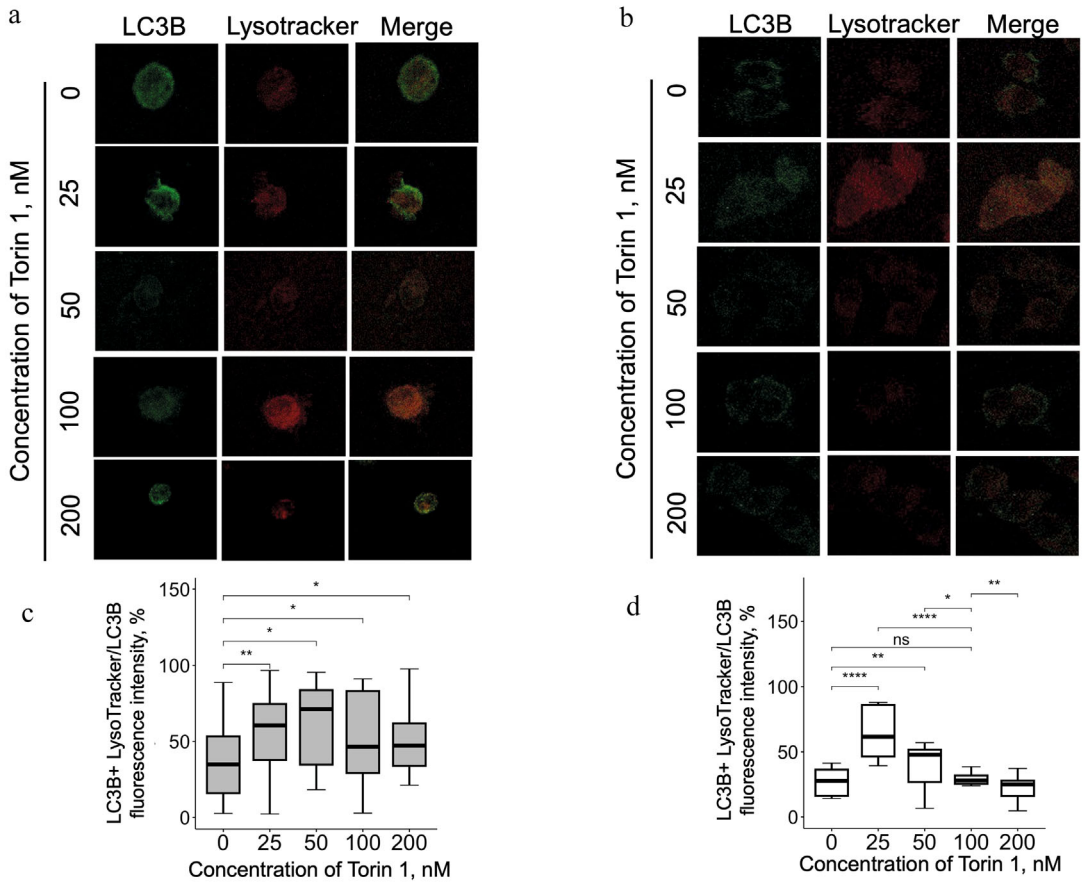

Fig. 3. Colocalization of LC3B with lysosomes upon Torin 1-mediated dose-dependent mTOR inhibition. a) LC3B protein and lysosome immunofluorescent staining in primary peripheral blood macrophages, 10 μm; b) LC3B protein and lysosome immunofluorescent staining in SH-SY5Y neuroblastoma cell line, 10 μm. c) colocalization of LC3B with lysosomes in primary peripheral blood macrophages (n = 6, where n is the number of independent samples); d) colocalization of LC3B with lysosomes in SH-SY5Y neuroblastoma cell line (n = 5, where n is the number of independent cell line samples). * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$; ns, not significant.

the LC3B-II level ($p > 0.05$; Fig. 2c). In contrast, in SH-SY5Y neuroblastoma cell line treated with Torin 1 at any dose did not significantly alter the LC3B-II protein level (Fig. 2e).

Furthermore, treatment of the primary peripheral blood macrophage culture derived from the neurologically healthy individuals with Torin 1 at all concentrations resulted in the higher degree of LC3B colocalization with lysosomes compared to intact cells ($p < 0.05$; Fig. 3c). Similarly, in the SH-SY5Y neuroblastoma cell line, exposure to Torin 1 at doses of 25 and 50 nM significantly increased LC3B colocalization with lysosomes compared to control cells ($p < 0.01$ and $p < 0.0001$, respectively; Fig. 3d).

Activity of the lysosomal enzymes (GCase, GLA, ASMase) and level of lysosphingolipids (HexSph, LysoGb3, LysoSM) involved in ceramide metabolism and associated with PD pathogenesis [28-30, 32, 33], were also assessed in primary peripheral blood macrophages derived from neurologically healthy individuals and in SH-SY5Y neuroblastoma cell line treated with autophagy inducer Torin 1 (Fig. 4). Interestingly, only at the 100-nM Torin 1 dose was there no significant effect on the lysosomal enzyme activity and lysosphingolipid level compared to the untreated cells ($p > 0.05$).

Moreover, it is worth mentioning that higher relative GCase protein level was discovered in primary peripheral blood macrophages derived from the neurologically healthy individuals (Fig. 2d) and in SH-SY5Y neuroblastoma cell line (Fig. 2f) after exposure to Torin 1 at a concentration of 100 nM ($p > 0.05$ and $p < 0.05$, respectively) compared to the untreated cells.

Torin 1-mediated dose-dependent mTOR inhibition affects the levels alpha-synuclein forms (monomeric, phosphorylated (Ser129), tetrameric) in the SH-SY5Y neuroblastoma cell line. In this study relative levels of different alpha-synuclein protein forms were assessed in SH-SY5Y neuroblastoma cell line (Fig. 5), but not in primary peripheral blood macrophages due to limitations in the sensitivity of the alpha-synuclein quantification assay. Treatment

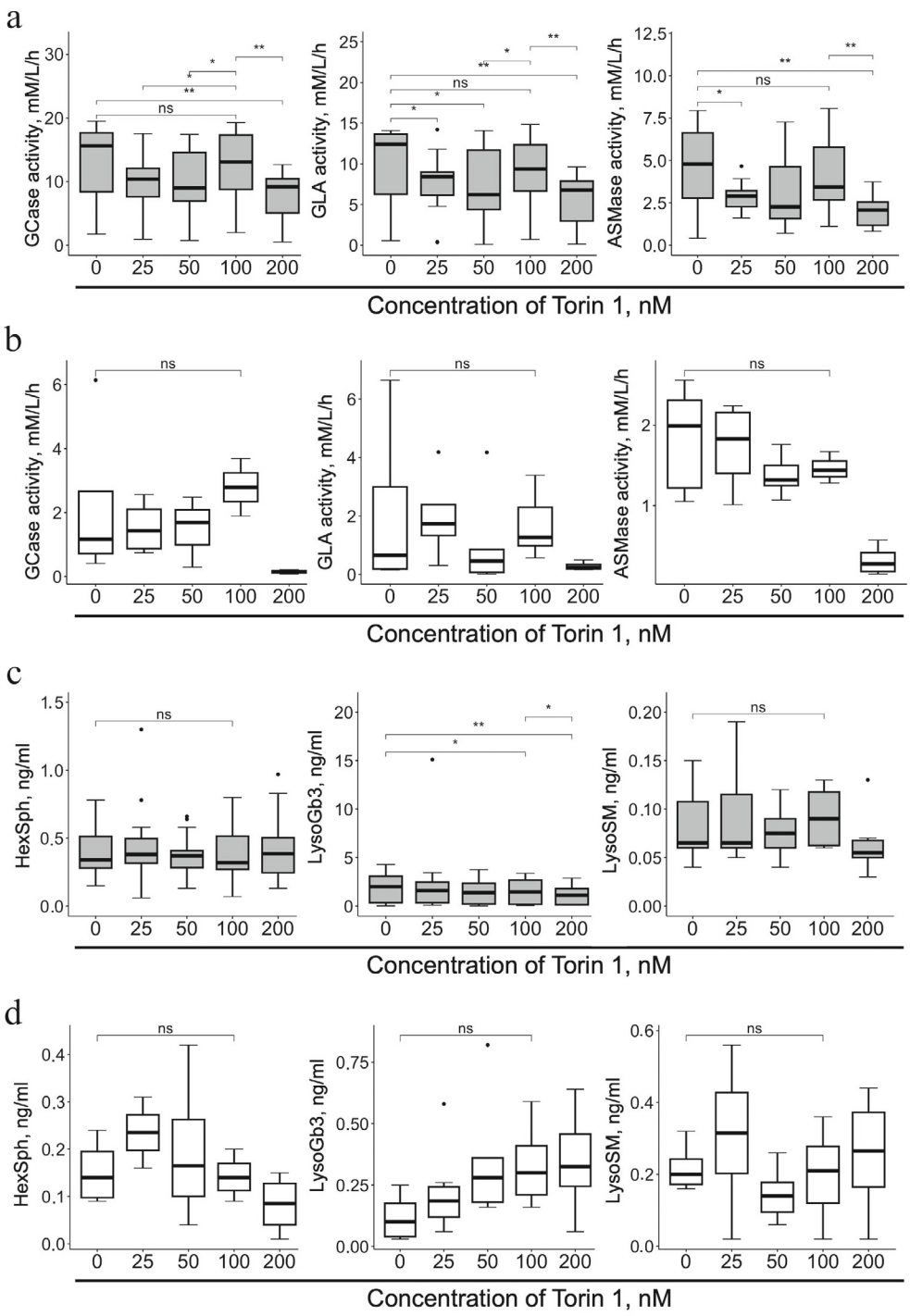

Fig. 4. Activity of lysosomal enzymes (GCase, GLA, ASMase) and levels of the relevant lysosphingolipid (HexSph, LysoGb3, LysoSM) upon Torin 1-mediated dose-dependent mTOR inhibition in primary peripheral blood macrophages (a and b; n = 6, where n is the number of independent samples) and in SH-SY5Y neuroblastoma cell line (c and d; n = 5, where n is the number of independent cell line samples). * $p < 0.05$; ** $p < 0.01$; ns, not significant.

of SH-SY5Y neuroblastoma cell line with Torin 1 at doses of 25, 100, and 200 nM resulted in a significant downmodulation of phosphorylated alpha-synuclein protein (Ser129) compared to untreated cells ($p < 0.01$; Fig. 5, a, b). Additionally, for the first time, elevated levels of tetrameric alpha-synuclein were detected in this cell line after treated with Torin 1 at concentrations of 50, 100, and 200 nM compared to untreated cells ($p < 0.01$; Fig. 5, a, d). In contrast, the level of monomeric alpha-synuclein protein remained unaltered in SH-SY5Y neuroblastoma cell line treated with Torin 1 ($p > 0.05$; Fig. 5, a and c).

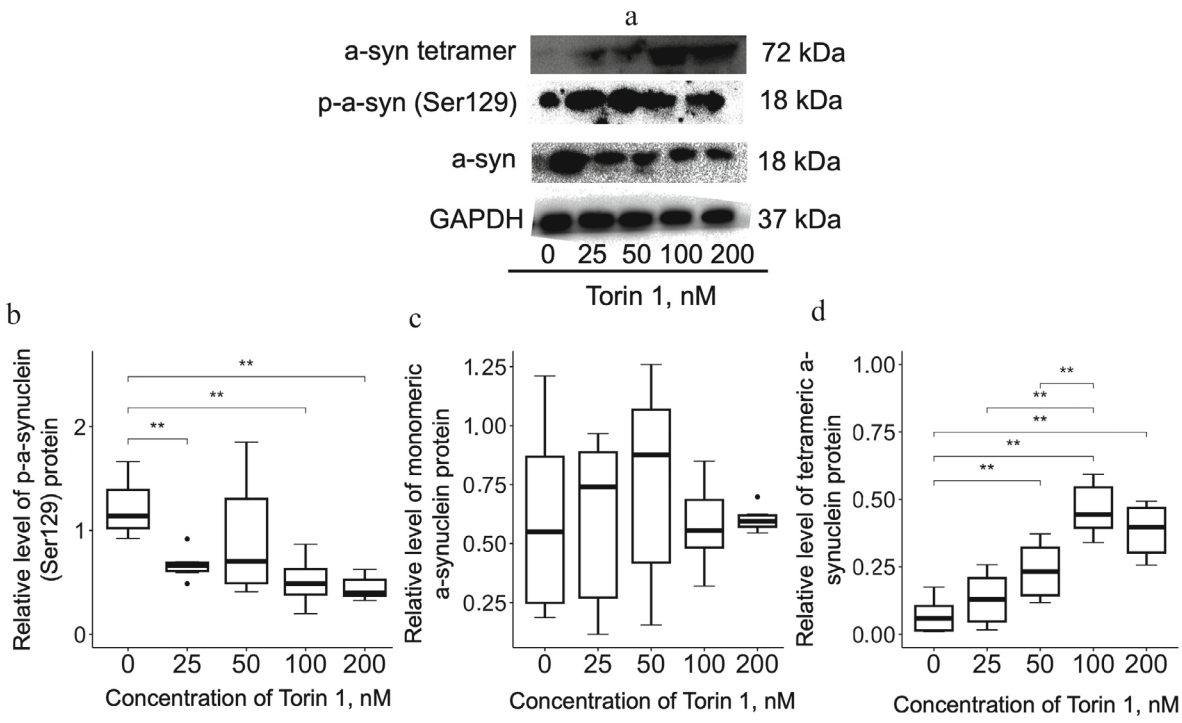

Fig. 5. Torin 1-mediated effect of dose-dependent mTOR inhibition on the levels of alpha-synuclein protein forms (monomeric, phosphorylated (Ser129), tetrameric) in SH-SY5Y neuroblastoma cell line (n = 5, where n is the number of independent cell line samples). a) Western blot data for alpha-synuclein (monomeric, phosphorylated (Ser129), tetrameric) forms. b) Relative level of phosphorylated (Ser129) alpha-synuclein protein; c) relative level of monomeric alpha-synuclein protein; d) relative level of tetrameric alpha-synuclein protein; ** $p < 0.01$.

DISCUSSION

Currently, no neuroprotective therapy capable of slowing down or stopping PD progression is available. The drugs used today only provide symptomatic effects. Hence, there is an urgent need to identify novel therapeutic targets for PD treatment, that may downregulate alpha-synuclein protein accumulation and reduce neuronal death. Recent evidence suggests a key role of lysosomal dysfunction and autophagy in PD pathogenesis [34, 35]. In this regard, proteins involved in or regulating autophagy events may be among the promising targets for developing PD therapy [36-38].

Here, we investigated the dose-dependent inhibitory effect of mTOR protein kinase essential for autophagy regulation on cellular parameters associated with PD pathogenesis Our findings revealed that mTOR inhibition after exposure to varying Torin 1 doses affects activity of the lysosomal hydrolases and level of the relevant substrates, lysosphingolipids, results in an increase in the GCase protein level in the primary peripheral blood macrophage culture and SH-SY5Y neuroblastoma cell line, as well as downregulates the level of phosphorylated alpha-synuclein (Ser129), while the level of its tetrameric form in SH-SY5Y neuroblastoma cell line exposed to autophagy inducer remains increased.

mTOR is an intracellular serine-threonine protein kinase comprising a subunit of the multimolecular signaling complexes mTORC1 and mTORC2, which, in turn, are components of PI3K/AKT/mTOR-axis. This axis plays a crucial role in controlling signal transduction and various biological events, such as cell proliferation, apoptosis, metabolism, angiogenesis, inflammation, as well as maintaining lysosomal function and autophagy [39, 40]. Alterations in the PI3K/AKT/mTOR signaling cascade that affects autophagy may lead to protein aggregate deposition and cell death in various proteinopathies including PD [41, 42]. In particular, changes in phosphorylated mTOR protein levels have been observed in the substantia nigra of mice with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced parkinsonism as well as in SH-SY5Y neuroblastoma cell line carrying missense-mutation A53T in the *SNCA* gene (the most common mutation in the *SNCA* gene causing early-onset autosomal-dominant PD. These changes increased mTOR/P70S6K signaling and disrupted autophagy, promoting further A53T alpha-synuclein aggregation [43-45]. Moreover, higher mTOR levels were found in the temporal cortex of brain autopsies of the patients suffering from dementia with Lewy bodies characterized by alpha-synuclein protein accumulation similar to PD patients [46]. Proteomic and Western blot analysis also showed elevated

phosphorylated mTOR protein levels in neurons differentiated from the induced pluripotent stem cells (iPSCs) obtained from the patients with GBA1-PD [11, 12]. Thus, mTOR may be a promising target for developing GBA1-PD therapy.

However, the data on inhibited mTOR protein kinase activity remain controversial, as both neuroprotective and neurotoxic effects were observed in various PD models. This variability could be related to mTOR imbalance, particularly due to the inhibitor doses used to elicit cell death [8, 19]. Currently, inhibitors of mTOR kinase activity are divided into four classes: antibiotic allosteric inhibitors selectively inhibiting mTORC1 complex (first generation inhibitors, rapamycin and its paralogs); ATP-competitive inhibitors capable of inhibiting both mTORC1 and mTORC2 complexes (second generation inhibitors, Ku-0063794, WYE-3541, Torin 1, etc.); dual mTOR/PI3K inhibitors (second generation inhibitors, GNE477, NVP-BEZ235, etc.); other new inhibitors (third generation inhibitors, P529, RapaLinks, etc.) [47]. Currently, direct and indirect inhibitors of mTOR kinase activity are undergoing clinical trials for assessing therapeutic effectiveness in diverse neurodegenerative diseases and proteinopathies [PD (NCT05357989, NCT05781711); Huntington's disease (NCT04826692); Alzheimer's disease (NCT03748706, NCT04511416); amyotrophic lateral sclerosis (NCT04577404)]. In our study, we chose the direct mTOR inhibitor Torin 1, which is not yet in clinical trials for neurodegenerative diseases. However, studies using mouse models of induced parkinsonism and cell lines from biallelic *GBA1* gene variant carriers (Gaucher disease) and patients with GBA1-PD have demonstrated that Torin 1 is effective with regard to the hallmark biochemical characteristics related to PD pathogenesis. These include the downregulation of phosphorylated alpha-synuclein (Ser129), restoration of autophagolysosomal system function and decrease of neurodegeneration [12, 23, 48], which allows considering it as a promising agent for PD therapy particularly for GBA1-PD.

In our study, the dose-dependent effect of mTOR kinase activity inhibition on lysosome functioning was discovered in cell models. This was evidenced by changes in level of essential autophagy marker LC3B-II, GCase protein, as well as altered activity of lysosomal hydrolases and levels of lysosphingolipids. Exposure of the primary peripheral blood macrophage culture to the mTOR inhibitor Torin 1 at doses of 25, 50, and 100 nM, and exposure of the SH-SY5Y neuroblastoma cell line to Torin 1 at doses of 25, 50, 100, and 200 nM, resulted in a slight increase in the relative level of LC3B-II protein. This was accompanied by an increased degree of LC3B protein colocalization with lysosomes in primary peripheral blood macrophage culture at all tested doses of Torin 1, and in SH-SY5Y neuroblastoma cell line at Torin 1 concentrations of 25 and 50 nM. Previously, it was shown that inhibition of the mTOR kinase activity in mouse models of MPTP-induced parkinsonism and in the neuroblastoma cell line treated with toxic 1-methyl-4-phenylpyridinium (MPP+) cations results in altered LC3 protein level and upregulated expression level of the main lysosome marker LAMP1, both at mRNA and protein levels [43, 49]. Torin 1-treated neurons derived from iPSC of GBA1-PD patients were also shown to have elevated LC3B-II protein level after exposure to chloroquine that inhibits autophagosomal degradation [12].

Activity of lysosomal hydrolases was assessed by analyzing the enzymes GCase, GLA, ASMase, as well as the level of sphingolipids HexSph, LysoGb3, LysoSM involved in ceramide metabolism, disruption of which is associated with PD pathogenesis [28-30, 32, 33]. The lysosomal enzymes GCase, GLA, ASMase are encoded by the *GBA1*, *GLA*, *SMPD1* genes, respectively. Mutations in these genes result in lysosomal storage diseases characterized by lower enzyme activity and accumulation of lysosphingolipids and also are considered as risk factors for PD development [50]. We discovered that activity of these enzymes decreases at all Torin 1 doses in the primary peripheral blood macrophage culture and SH-SY5Y neuroblastoma cell line, except for the 100-nM concentration, when the enzyme activities and substrate levels remained unchanged similar to those in the cells without the autophagy inducer. Hence, it suggests that exposure to Torin 1 at doses of 25, 50, and 200 nM could cause imbalance in cellular processes particularly affecting lysosome functioning, which may eventually trigger cell death [8, 19]. Apart from this, we assessed for the first time the effect of Torin 1 on the relative GCase enzyme level, activity of which is decreased in GBA1-PD and sPD [51-53]. We found that the relative GCase protein level increased in the primary peripheral blood macrophage culture and in the SH-SY5Y neuroblastoma cell line after exposure to Torin 1 at concentrations of 25 and 100 nM, and 50, 100, and 200 nM, respectively. Previously, another inhibitor, RTB101, a dual mTOR/PI3K inhibitor, was shown to decrease the level of glucosylceramide, an essential GCase substrate, both in blood and cerebrospinal fluid of GBA1-PD patients [54].

It is interesting to note that mTOR-dependent autophagy inducers were also previously shown to affect the level of alpha-synuclein protein that plays an essential role in PD pathogenesis [12, 17, 18]. Various alpha-synuclein forms exist in cells. The phosphorylated alpha-synuclein (Ser129) promotes its aggregation and exhibits the highest cell toxicity. This form is most often found in pathological inclusions in PD [55]. In contrast, the tetrameric form of alpha-synuclein is considered physiological and more stable, whereas the monomeric form is prone to forming neurotoxic

oligomers [56]. In this study, we observed decline in the level of phosphorylated alpha-synuclein protein (Ser129) without changes in the monomeric form in SH-SY5Y neuroblastoma cell line treated with Torin 1 at doses of 25, 100, and 200 nM compared to the intact cells. Previously, a dose-dependent reduce in phosphorylated (Ser129) and monomeric alpha-synuclein forms was shown in cell and animal models overexpressing alpha-synuclein after mTOR inhibition [17, 18, 57, 58]. In addition, neurons derived from iPSC-of GBA1-PD patients showed a slight downregulation of phosphorylated alpha-synuclein (Ser129) after Torin 1 exposure [12]. The mechanism underlying this decline in the level of phosphorylated alpha-synuclein protein due to Torin 1-mediated mTOR inhibition remains unknown. However, suppression mTOR activity by metformin, a second-generation inhibitor, like Torin 1, was shown to activate protein phosphatase 2A (PP2A) that may dephosphorylate, alpha-synuclein, in primary murine hippocampal neuron culture [18]. At the same time, we were the first to demonstrate an increased level of tetrameric alpha-synuclein form in SH-SY5Y neuroblastoma cell line upon exposure to Torin 1 at all doses examined compared to the control cells.

The current study has several limitations. The primary peripheral blood macrophage culture was obtained from neurologically healthy individuals and SH-SY5Y neuroblastoma cell line was used without induced parkinsonism and GCase enzyme dysfunction. Further studies with patient-specific cells derived from patients with PD, particularly GBA1-PD, as well as the cell lines that accurately model the disease, are required.

CONCLUSION

Despite previous demonstrations of the neuroprotective properties of Torin 1 in animal and cell models, our study using primary peripheral blood macrophage culture derived from the neurologically healthy individuals and SH-SY5Y neuroblastoma cell line provides deeper insights into the effect of mTOR inhibitor Torin 1 on altering cell parameters, which may be related to PD. In this regard, we demonstrated that exposure to Torin 1 at different doses may decrease lysosomal hydrolase activities and elevate lysosphingolipid concentrations, which could be fatal to the cells. However, optimal doses of Torin 1 may induce autophagy, increase GCase protein level, decrease phosphorylated alpha-synuclein (Ser129) level, and increase its tetrameric form without significantly affecting lysosomal hydrolase activities and lysosphingolipid levels. Torin 1-mediated mTOR protein kinase inhibition shows promise for developing therapies for PD, particularly for the GBA1-PD. Determining therapeutic dosages of Torin 1 will be crucial. Such studies are very important for expanding our understanding of the molecular mechanisms of various chemicals and assessing their potential clinical applications.

Contributions. T.S.U. conceptualized and supervised the study; A.I.B., K.S.B., and G.V.B. conducted the experiments; A.I.B., E.Y.Z., S.N.P., and T.S.U. discussed the study data; A.I.B. wrote the manuscript; T.S.U. edited the manuscript.

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Ethics declarations. All the procedures carried out in the research with participation of humans were in accordance with the ethical standards of the National Research Ethics Committee and with the Helsinki Declaration of 1964 and its subsequent changes or with comparable ethics standards. Informed voluntary consent was obtained from every participant of the study. The study was approved by the ethics committee of the Pavlov First Saint Petersburg State Medical University (protocol no. 275 dated of 09/04/2023). The authors declare that they no conflicts of interest.

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