CLINICAL-DIAGNOSTIC STUDIES

SIMULTANEOUS INHIBITION OF mTOR AND STING AS AN APPROACH TO REDUCE ALPHA-SYNUCLEIN AND LYSOSPHINGOLIPID LEVELS IN PERIPHERAL BLOOD MONOCYTE-DERIVED MACROPHAGES AND THE SH-SY5Y CELL LINE: IMPLICATIONS FOR THERAPY OF PARKINSON'S DISEASE

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The combined effects of two inhibitors, Torin 1, acting on mTOR, a key regulator of autophagy, and H-151, inhibiting STING, a key regulator of inflammation, on the autophagolysosomal system, have been studied in a primary culture of peripheral blood macrophages from healthy donors and the SH-SY5Y neuroblastoma cell line. Combined use of these drugs resulted in a decrease in the levels of lysosphingolipids, triggering alpha-synuclein oligomerization, as well as a decrease in the levels of monomeric and neurotoxic phosphorylated (Ser129) alpha-synuclein and an increase in tyrosine hydroxylase. These results open new prospects for the use of combination therapy with these proposed drugs in the treatment of both diseases associated with lysosomal dysfunction and neurodegenerative pathologies.

Keywords: Parkinson's disease; mTOR; STING; alpha-synuclein; lysosomal hydrolase activity; autophagy

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INTRODUCTION

Parkinson's disease (PD) is a chronic, progressive, and widespread neurodegenerative disorder characterized by dopaminergic neurons cell death in the *substantia nigra* of the human brain [1–4]. The pathogenesis is based on the accumulation and aggregation of the alpha-synuclein protein [5]. Although the molecular mechanisms of PD remain unknown, recent studies indicate an important role of inflammatory processes and autophagy disorders in the pathogenesis of the disease [6–10]. Chronic inflammation leads to immune cell activation, which increases the vulnerability of neurons to degeneration [11]. Since autophagy is involved in the degradation of defective and toxic proteins, including alpha-synuclein, impairments in the autophagolysosomal system functioning also play a significant role in the PD pathogenesis [12]. It is known that hyperactivation of the mTOR signaling pathway leads to suppression of autophagy, which contributes to the accumulation of proteins, including alpha-synuclein, and the progression of neurodegeneration processes [13–15]. In addition, activation of inflammatory processes can further suppress autophagy through signaling pathways, including cGAS-STING [16]. We and other reseachers have shown an increase in the secretion of proinflammatory cytokines in blood plasma and mTOR signaling disruption in cells of patients with sporadic PD (sPD) and patients with the most common form of PD with a known etiology: PD associated with mutations in the *GBA1* gene (GBA1-PD) [9, 17–22].

A recently registered clinical trial of cilostazol as a drug for treatment for PD (NCT06612593), which simultaneously modulates the mTOR and STING pathways through antioxidant and anti-inflammatory

Abbreviations used: ASMase – acidic sphingomyelinase; GALC – galactocerebrosidase; GBA1-PD – Parkinson's disease associated with mutations in the *GBA1* gene; GCase -–glucocerebrosidase; GLA – alpha-galactosidase; HexSph – hexasylsphingosine; i-CTSD – intermediate form of the cathepsin D protein; LysoGb3 – lysoglobotriaosylsphingosine; LysoSM – lysosphingomyelin; m-CTSD – mature form of cathepsin D; pro-CTSD – pro-form of cathepsin D; TH – tyrosine hydroxylase; iPSC – induced pluripotent stem cells; PD – Parkinson's disease; sPD – sporadic Parkinson's disease.

effects, confirms the relevance of this combination approach. Simultaneous modulation of these two signaling cascades may be a promising therapeutic strategy for PD treatment, resulting in a synergistic effect. However, the cellular response to combined inhibition of these signaling pathways remains unknown.

In this study, we have evaluated for the first time the combined effects of a selective inhibitor of mTOR (Torin 1), a key regulator of autophagy, and a selective inhibitor of STING (H-151), a key regulator of inflammation, on autophagolysosomal system parameters, particularly, lysosomal enzyme activity and lysosphingolipid concentrations, levels of key protein markers of autophagy, levels of various forms of alpha-synuclein, and the degree of apoptosis in a primary culture of human peripheral blood macrophages and the SH-SY5Y neuroblastoma cell line.

Macrophages play a key role in regulation of the inflammatory response, autophagy, and apoptosis [23], and the SH-SY5Y cells are widely used to study the mechanisms of alpha-synuclein degradation, autophagy, and apoptosis [24].

MATERIALS AND METHODS

Characteristics of the Patients Included in the Study

The study included 10 neurologically healthy individuals (3 men, 7 women, average age 27.0±5.5 years) who were observed at the Consultative and Diagnostic Center of the First Pavlov State Medical University of St. Petersburg.

Cultivation of a Primary Culture of Peripheral Blood Macrophages

A primary culture of peripheral blood macrophages was obtained from peripheral blood mononuclear cells of each individual, according to the protocol described previously [25–27]. On day 4, the selective mTOR protein kinase inhibitor Torin 1 (Abcam, USA), the STING protein inhibitor H-151 (MCE, Sweden) or combination of these inhibitors were added to the primary macrophage culture. Final concentrations of Torin 1 (25 nM, 50 nM, 100 nM, 200 nM), H-151 (0.25 μM, 0.5 μM, 1 μM, 2 μM) and their combinations (50 nM, 100 nM Torin 1 and 0.5 μM, 1 μM H-151, respectively) were selected based on the results of the survival assessment [28]. After Torin 1 and/or H-151 addition the primary macrophage culture was cultivated for 24 h.

Cultivation of the SH-SY5Y Neuroblastoma Cell Line

The SH-SY5Y neuroblastoma cells, kindly provided by colleagues from the Institute of Cytology, Russian Academy of Sciences, St. Petersburg (Dr Biol. Sci. E.V. Kaznacheeva) were cultured in DMEM nutrient medium (Biolot, Russia) supplemented with 10% fetal bovine serum (Biolot) and 1% gentamicin (Biolot) for 4 days at 37°C and 5% CO_2. Differentiation of SH-SY5Y cells was performed according to the protocol described previously. On day 9 of cultivation, the selective mTOR protein kinase inhibitor Torin 1 (25 nM, 50 nM, 100 nM, 200 nM) or the STING inhibitor H-151 (0.25 μM, 0.5 μM, 1 μM, 2 μM) or a combination of Torin 1 and H-151 inhibitors (50 nM, 100 nM, and 0.5 μM, 1 μM, respectively), determined on the basis of survival assessment data, were added to SH-SY5Y neuroblastoma cells. Cultivation continued for 24 h. Each experiment was performed in 5 independent replicates.

Determination of Protein Levels of Phosphorylated mTOR, Phosphorylated RPS6, STING, Phosphorylated TBK1, BECN1, p62, LC3B, CTSD, GCase, Tyrosine Hydroxylase, and alpha-Synuclein by Western Blotting

Total protein was quantified using the BCA Protein Quantitative Detection Kit (ServiceBio, China). Equal amounts of protein were separated by polyacrylamide gel electrophoresis (20% SDS-PAGE for LC3B, 12% SDS-PAGE for other proteins) and transferred to a polyvinylidene fluoride membrane (Bio-Rad, USA). Measurements of the protein levels of the phosphorylated form of mTOR (Ser2448) (p-mTOR), phosphorylated form of RPS6 (Ser235/236) (p-RPS6), STING, phosphorylated form of TBK1 (Ser172) (p-TBK1), BECN1, p62, LC3B, CTSD, GCase, tyrosine hydroxylase (TH) and alpha-synuclein (phosphorylated (Ser129), monomeric and tetrameric forms) in primary cultured macrophages of the peripheral group of neurologically healthy individuals and the SH-SY5Y neuroblastoma cell line in the presence of inhibitors of mTOR protein kinase and/or STING protein used at various concentrations described above and without inhibitors were performed using the appropriate primary antibodies (dilution 1:1000) (Phospho-mTOR-S2448, ABclonal (USA), AP0094; Phospho-RPS6-S235/236, ABclonal, AP1326; STING, Cloud-Clone Corp. (China), PAN011Hu01; Phospho-TBK1-S172, Affinity Biosciences (China), AF8190; BECN1, Cloud-Clone Corp., PAJ557Hu01; p62, Cloud-Clone Corp., PAD198Hu01; LC3B, ABclonal, A19665; CTSD, Cloud-Clone Corp., PAB280Hu01; GCase, ABclonal, A19057; TH, Cloud-Clone Corp., PAB438Hu01; Phospho-α-Synuclein (Ser129) (D1R1R), Cell Signal (USA), 23706; anti-alpha-synuclein oligomeric, Sigma (USA), ABN2265). Abcam antibodies conjugated with horseradish peroxidase (goat anti-rabbit HRP conjugate, Abcam, ab6721; dilution of 1:5000) were used

as secondary antibodies. Secondary antibodies were stained using the Clarity Western ECL Blotting Substrate detection system (BioRad). The amount of the studied protein was normalized to the corresponding values for the reference protein GAPDH (ABclonal, AC036, 1:15000). For each protein, the experiments were performed in triplicate. Western blotting results were analyzed using the Fiji software (version 2.14.0/1.54f).

Evaluation of Lysosomal Enzyme Activities and Lysosphingolipid Concentrations by High-Performance Liquid Chromatography with Tandem Mass Spectrometry

The enzymatic activity of lysosomal enzymes (GCase, alpha-galactosidase (GLA), acidic sphingomyelinase (ASMase), galactocerebrosidase (GALC)) and the concentrations of the corresponding substrates (hexasylsphingosine (HexSph) (a mixture of glycosylsphingosine (GlcSph) and galactosylsphingosine (GalSph)), lysosphingomyelin (LysoSM), and lysoglobotriaosylsphingosine (LysoGb3)) were measured by high-performance liquid chromatography with tandem mass spectrometry (HPLC-MS/MS) in a primary culture of peripheral blood macrophages from a group of neurologically healthy individuals and a SH-SY5Y neuroblastoma cell line. Cells were cultivated in the presence or in the absence of mTOR protein kinase (Torin 1) and/or STING (H-151) inhibitors at the concentrations described above, and using the previously described protocol [29, 30]. All measurements were performed in triplicate.

The enzymatic activity of GCase, GLA, ASMase, and GALC was assessed by measuring the concentration of the product formed during the enzyme-catalyzed substrate conversion. Measurements were performed on an API 3200 QTrap tandem mass spectrometer (ABSciex, USA) in the multiple reaction monitoring (MRM) mode. Activity was calculated assuming that the amount of product formed was directly proportional to the enzyme activity in the cell suspension. Samples with known enzyme activity obtained from the Centers for Disease Control and Prevention (Atlanta, USA) were included in each plate as controls.

Lysosphingolipids HexSph, LysoSM, and LysoGb3 were extracted from dried spots of cell suspension by adding 100 μl of extraction solvent (80% methanol, 15% acetonitrile, and 5% water) containing 10 ng/ml IS (LysoLC), followed by incubation for 60 min (at 30°C, 650 rpm). The extracted lysosphingolipids were transferred to a new 96-well plate. The HPLC-MS/MS system consisted of a Shimadzu Nexera HPLC (Shimadzu Corporation, Japan) and an API-5500 QTrap mass spectrometer (ABSciex). Separation of metabolites was performed on a Phenomenex Fusion-RP column (Phenomenex, USA) 4 μm 2.1×50 mm in linear gradient mode.

Assessment of Apoptosis in Primary Cultures of Peripheral Blood Macrophages using Flow Cytometry

Apoptosis in primary cultured peripheral blood macrophages from neurologically healthy individuals in the absence and in the presence of concentrations of mTOR protein kinase and/or STING protein inhibitors described above, was assessed by flow cytometry using the Annexin V-FITC/PI Apoptosis Detection kit (Kit A211, Vazyme, China) according to the manufacturer's protocol on a Cytomics FC-500 flow cytometer (Beckman Coulter, USA).

Statistical Data Processing

Statistical data processing was performed using the built-in R package (version 4.5.2) [31]. The Kruskal-Wallis test was used to assess differences between several groups. The Wilcoxon paired test was used to assess differences between groups. Differences were considered statistically significant at $p < 0.05$. Clinical characteristics are given as mean ± standard deviation, experimental values as median (min–max). To quantify the interaction between Torin 1 and H-151, the Highest Single Agent (HSA) method was used. Cellular response data (marker levels) were converted into a dose-response matrix specifying Torin 1 and H-151 concentrations, including monotherapy and zero doses (control). Calculation and visualization of the synergistic effect were performed using the SynergyFinder package in R and further verified using the SynergyFinder Plus online platform.

RESULTS

Currently, the modern trends in PD therapy development include not only the search for new therapeutic targets but also the study of combination approaches to achieve a synergistic effect with low drug doses and to reduce the risk of adverse effects rather than. In this context, a strategy for simultaneous targeting of several key signaling pathways involved in the pathogenesis of PD is particularly important. In this study, we have assessed the dose-dependent and combined effects of mTOR (Torin 1) and STING (H-151) inhibitors on key parameters of the autophagolysosomal system, including lysosomal enzyme activity, lysosphingolipid concentrations, autophagy levels, various forms of alpha-synuclein and GCase, and the degree of apoptosis in primary cultures of human peripheral blood macrophages and the SH-SY5Y neuroblastoma cell line.

The main part of the study presents data on the effects of Torin 1 (50 nM, and 100 nM) and H-151 (0.5 μM and 1 μM) inhibitors separately, as well as their combinations at the same concentrations, on all studied parameters. The Supplementary Materials show dose-dependent changes over a wider range of Torin 1 concentrations: 25 nM, 50 nM, 100 nM, and 200 nM, and H-151: 0.25 μM, 0.5 μM, 1 μM, and 2 μM (Supplementary Materials, Figs. S1–S10).

Evaluation of the Efficiency of mTOR and STING Inhibition in Primary Cultures of Peripheral Blood Macrophages from Neurologically Healthy Individuals and the SH-SY5Y Neuroblastoma Cell Line in the Presence of Torin 1 and/or H-151

The efficiency of mTOR protein kinase and STING inhibition by the corresponding Torin 1 and H-151 inhibitors in macrophages and SH-SY5Y cells was assessed based on the levels of p-mTOR (Ser2448) and p-RPS6 (Ser235/236) (for Torin 1) (Fig. 1A), STING, and p-TBK1 (Ser172) (for H-151) (Fig. 2A).

Phosphorylated forms of proteins have been selected as markers of signaling pathway activity because the degree of their phosphorylation reflects the activation state of corresponding molecular cascades. The phosphorylated form of mTOR kinase (p-mTOR, Ser2448) reflects its catalytic activity; phosphorylation at Ser2448 is associated with activation of the mTOR complex, which regulates cell growth, metabolism, and protein synthesis. A decrease in the p-mTOR level at Ser2448 induced by its inhibitor, Torin 1, indicates suppression of the mTOR complex activity. Ribosomal protein S6 (RPS6) is phosphorylated by S6K kinase, which is a direct substrate of mTOR; therefore, the level of p-RPS6 (Ser235/236) is considered as a secondary indicator of the mTOR pathway activity. A decrease in the level of p-RPS6 (Ser235/236) induced by the treatment with Torin 1 further confirms the effectiveness of inhibition of this signaling cascade. The STING (Stimulator of Interferon Genes) protein is a key adaptor of the innate immune response, activating the TBK1 kinase and the IRF3 transcription factor. Activation of the STING protein is accompanied by conformational changes and the initiation of phosphorylation of downstream effectors of the signaling cascade. The autophosphorylated form of the serine/threonine kinase TBK1 at Ser172 reflects its active state and serves as an indicator of functional activity of the STING signaling. The inhibitor H-151 prevents STING activation by blocking its palmitylation, which leads to a decrease in p-TBK1 levels. Thus, phosphorylated forms of p-mTOR and p-RPS6 have been used to assess the effectiveness of mTOR pathway inhibition, while p-TBK1 levels have been used to assess the suppression of STING-dependent activation.

The combined effect of the inhibitors was compared with their individual effects, as well as with untreated cell cultures.

We have shown previously [32], that Torin 1 (50 nM and 100 nM) insignificantly influenced the level of p-mTOR (Ser2448) compared to the untreated primary culture of peripheral blood macrophages from healthy donors (Fig. 1B). However, treatment with H-151 (0.5 μM and 1 μM) led to a statistically significant decrease in the level of p-mTOR (Ser2448) compared to the untreated cell culture ($p < 0.05$) (Fig. 1B). The combined effect of Torin 1 (50 nM and 100 nM) and H-151 (1 μM) led to an increase in the level of p-mTOR (Ser2448) compared to the effect of H-151 used at the same concentration ($p < 0.05$) (Fig. 1B). In SH-SY5Y cells, mTOR inhibition by Torin 1 (100 nM) caused a statistically significant decrease in the p-mTOR (Ser2448) level compared to untreated cells ($p < 0.001$) (Fig. 1B), as we showed previously [32]. However, the combined addition of H-151 (0.5 μM) and Torin 1 (50 nM) caused an increase in p-mTOR (Ser2448) compared to untreated cells and the monodrugs at the same concentrations (Fig. 1B) thus suggesting a cross-regulatory effect. A similar effect, expressed as an increase in p-mTOR (Ser2448), was observed when cultured cells were treated with a combination of Torin 1 (100 nM) and H-151 (1 μM) as compared to the effect of Torin 1 alone (100 nM) (Fig. 1B).

The level of p-RPS6 (Ser235/236) protein as a downstream target of mTOR in the primary culture of peripheral blood macrophages treated with Torin 1 (50 nM and 100 nM) decreased in a dose-dependent manner as compared to the cell culture without the addition of inhibitors ($p < 0.001$) (Fig. 1C). However, the combined treatment of cells with Torin 1 and H-151 in all studied combinations led to an increase in the level of p-RPS6 (Ser235/236) as compared to the effect of Torin 1 alone (50 nM and 100 nM) (Fig. 1C). The combination of Torin 1 (100 nM) and H-151 (0.5 μM) led to a more pronounced increase in p-RPS6 (Ser235/236) in the primary culture of peripheral blood macrophages compared to the addition of only H-151 (0.5 μM) and in the absence of inhibitors ($p < 0.05$) (Fig. 1C).

In SH-SY5Y cells, a decrease in the relative level of p-RPS6 (Ser235/236) was observed after treatment with Torin 1 (50 nM and 100 nM) in a dose-dependent manner and with H-151 (1 μM) alone as compared to untreated cell culture. The combination of Torin 1 (50 nM) with H-151 (0.5 μM and 1 μM) and Torin 1 (100 nM) with H-151 (0.5 μM) resulted in an increase in p-RPS6 (Ser235/236) versus Torin 1 (50 nM and 100 nM) alone ($p < 0.05$) (Fig. 1C). A decrease in p-RPS6 (Ser235/236) levels was detected in the case of the combination of Torin 1 with H-151

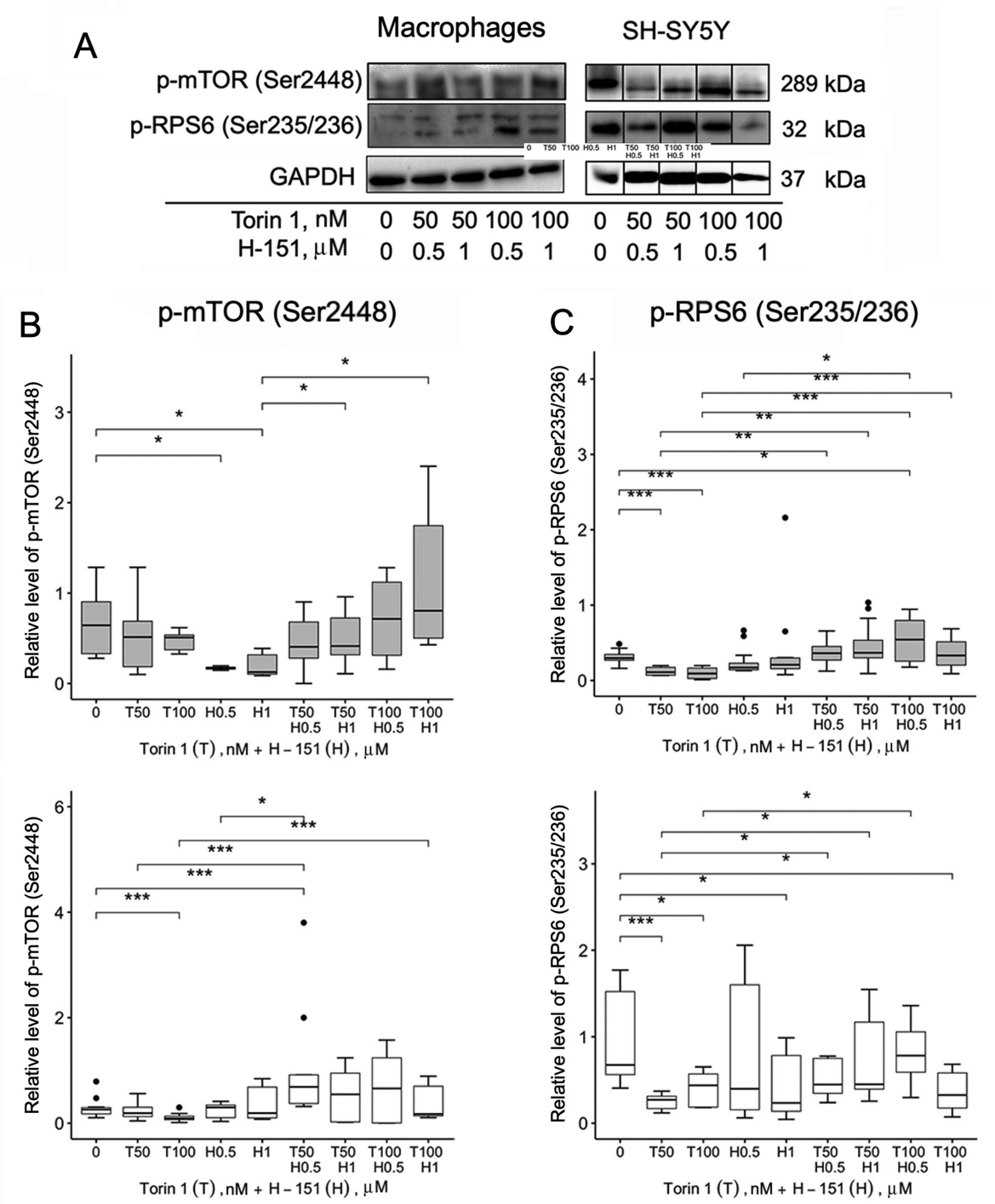

Figure 1. Evaluation of the efficacy of combined inhibition of the mTOR protein kinase by Torin 1 and H-151 in primary cultures of peripheral blood-derived macrophages (macrophages; gray bars) and SH-SY5Y neuroblastoma cells (SH-SY5Y; white bars). **A** – Western blotting data; **B** – relative level of p-mTOR (Ser2448); **C** – relative level of p-RPS6 (Ser235/236). T – Torin 1, H – H-151. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

used at the highest concentrations employed in this study as compared to untreated cell culture ($p < 0.05$) (Fig. 1C).

STING levels were reduced in primary macrophage cultures treated with a combination of Torin 1 (50 nM) and H-151 (1 μM) compared to untreated cells ($p < 0.05$); the same trend was observed in the presence of a combination of Torin 1 (100 nM) and H-151 (0.5 μM) as compared to the STING level in cells treated with the same concentration H-151 ($p < 0.05$) (Fig. 2B).

In the case of SH-SY5Y cells addition of Torin 1 (50 nM) reduced the relative STING level compared to inhibitor-untreated cells ($p < 0.05$) (Fig. 2B). At the same time, combined use of the inhibitors (Torin 1 and H-151) at the maximal

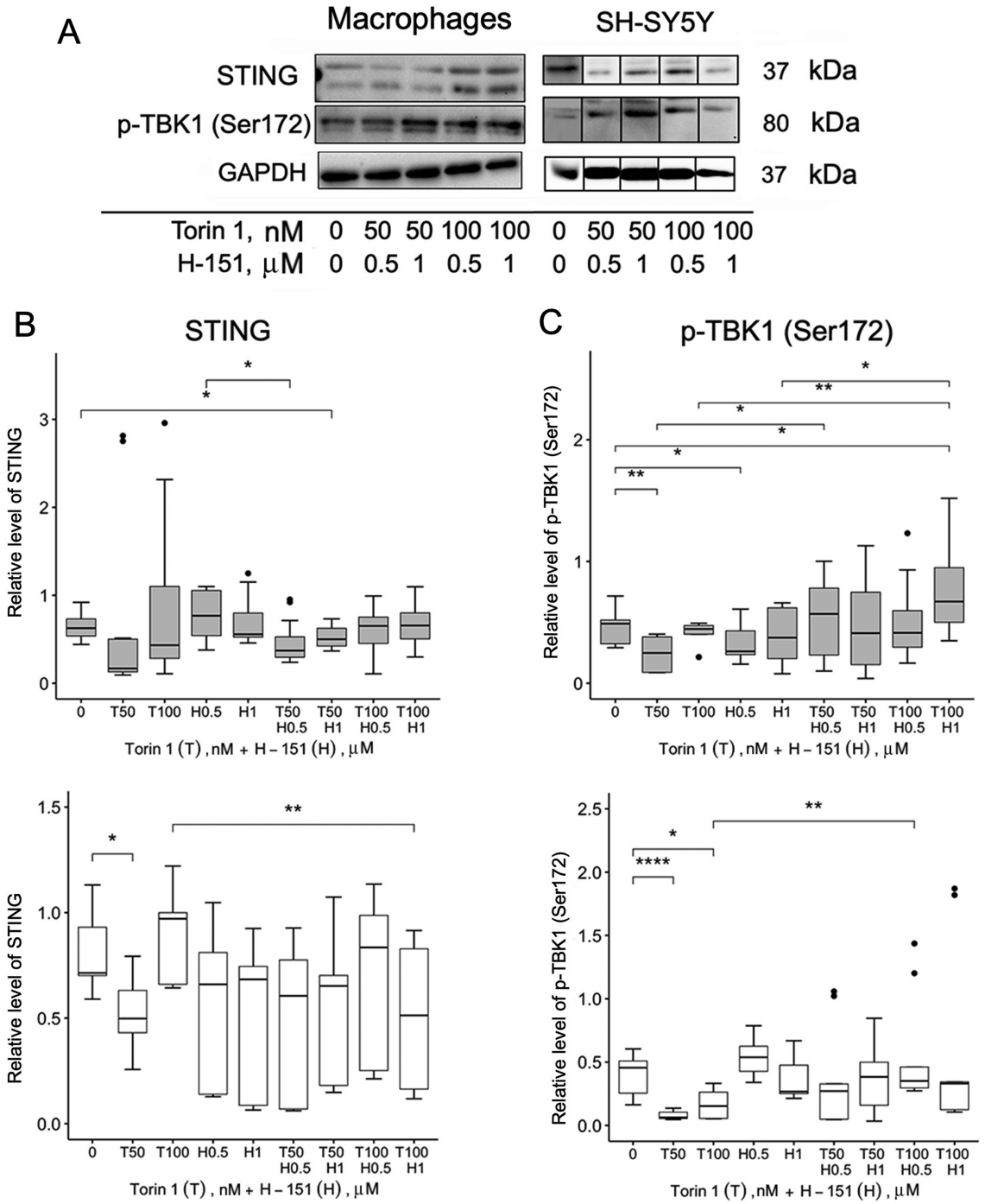

Figure 2. Evaluation of the efficacy of combined inhibition of the STING protein by Torin 1 and the H-151 in primary cultures of peripheral blood-derived macrophages (macrophages; gray bars) and SH-SY5Y neuroblastoma cells (SH-SY5Y; white bars). **A** – Western blotting data; **B** – relative level of STING; **C** – relative level of p-TBK1 (Ser172). T – Torin 1, H – H-151. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

concentrations studied resulted in a decrease in STING as compared to Torin 1 alone (100 nM) ($p < 0.01$) (Fig. 2B).

The level of p-TBK1 (Ser172), a key mediator in the downstream STING pathway, showed divergent changes in response to both mono- and combined treatments with inhibitors. For example, in the primary culture of macrophages, low concentrations of Torin 1 inhibitors (50 nM) and H-151 (0.5 μM) added separately, reduced the level of p-TBK1 (Ser172) as compared to the untreated cell culture, while a combination of high concentrations of Torin 1 (100 nM) and H-151 (1 μM) caused an increase in its level compared to the untreated cell culture and monotherapy by Torin 1 and H-151 at the same concentrations; combined treatment with low concentrations of Torin 1 and H-151 caused the same effect as compared to Torin 1 (50 nM)

($p < 0.05$) (Fig. 2C). In SH-SY5Y cells, Torin 1 (50 nM and 100 nM) decreased p-TBK1 (Ser172) levels compared to untreated cells, while H-151 alone and combination with Torin 1 had no effect on p-TBK1 (Ser172) levels compared to untreated cells ($p > 0.05$) (Figure 2C). However, the combination of Torin 1 (100 nM) and H-151 (0.5 μM) increased p-TBK1 (Ser172) levels compared to Torin 1 alone used at the same concentration ($p < 0.01$) (Fig. 2C).

These results suggest that combined inhibition of the mTOR and STING pathways by Torin 1 and H-151 has complex and dose-dependent effects on the key proteins of the signaling cascades studied.

Combined Effects of the mTOR Inhibitor Torin 1 and the STING Inhibitor H-151 on Key Autophagy Stages in Primary Cultures of Peripheral Blood Macrophages from Neurologically Healthy Individuals and the SH-SY5Y Neuroblastoma Cell Line

Autophagy is a process of cell degradation and recycling of dysfunctional molecules and organelles; it is highly conserved across all eukaryotes. The autophagy process can be divided into several stages: phagophore formation and elongation (BECN1), autophagosome formation and maturation (LC3B-II and p62), and autolysosome synthesis and degradation of its contents (Fig. 3A).

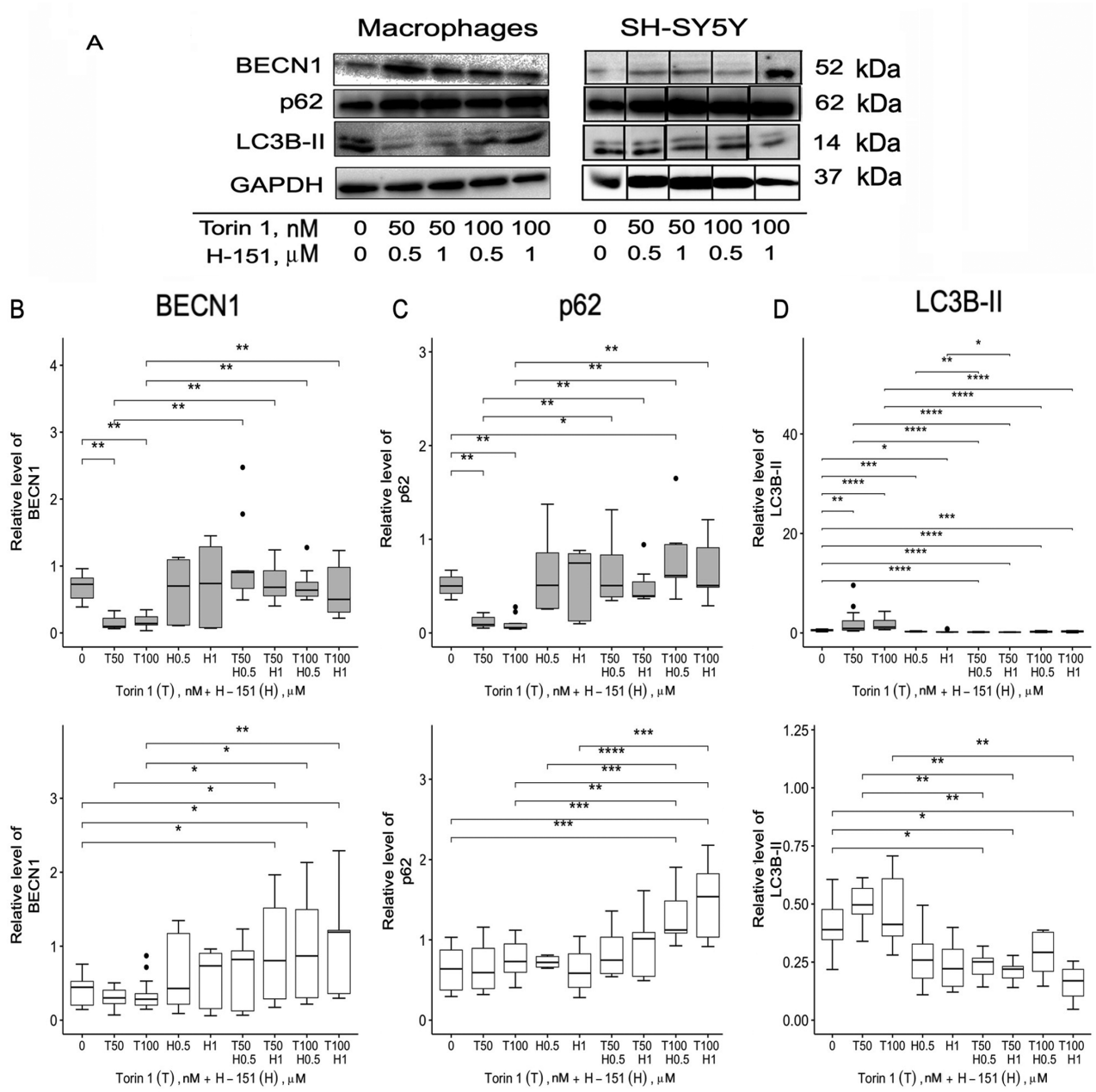

Figure 3. Evaluation of the dose-dependent combined effects of mTOR inhibition by Torin 1 and STING inhibition by H-151 in primary cultures of peripheral blood-derived macrophages (macrophages; gray bars) and the SH-SY5Y neuroblastoma cells (SH-SY5Y; white bars) on key stages of autophagy. **A** – Western blotting data; **B** – relative level of BECN1; **C** – relative level of p62; **D** – relative level of LC3B-II. T – Torin 1, H – H-151. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

In the primary culture of peripheral blood macrophages, Torin 1 (50 nM and 100 nM) decreased the levels of BECN1 and p62 and increased LC3B-II as compared to untreated cell culture ($p < 0.01$), thus reflecting the activation of autophagy and, in particular, the formation of autophagosomes (Fig. 3B–D), which was partially demonstrated in our previous study [32]. Interestingly, all studied combinations of Torin 1 (50 nM and 100 nM) and H-151 (0.5 μM and 1 μM) led to the opposite effect: they caused an increase in BECN1 and p62 (under conditions of a LC3B-II decrease) as compared to a single effect of Torin 1 (50 nM and 100 nM) ($p < 0.01$) (Fig. 3B–D).

However, cultivation of peripheral blood macrophages in the presence of H-151 (0.5 μM and 1 μM), both in combination with Torin 1 (50 nM and 100 nM) and alone, decreased the LC3B-II level ($p < 0.05$) (Fig. 3D) as compared to cells cultivated without inhibitors, while the combination of Torin 1 (100 nM) and H-151 (0.5 μM) increased the p62 level ($p < 0.05$) (Fig. 3C). The lack of changes in the BECN1 level of cells treated with any combination of Torin 1 and H-151 and single exposure to H-151 ($p > 0.05$) compared to untreated cell culture (Fig. 3B) may indicate a blockade of autolysosome degradation. The combination of Torin 1 (50 nM) and H-151 (0.5 μM and 1 μM) reduced LC3B-II levels compared to H-151 alone at the corresponding concentrations (Fig. 3D).

In the SH-SY5Y neuroblastoma cells, various combinations of Torin 1 and H-151 increased BECN1 and p62 levels ($p < 0.05$) (Fig. 3B,C) but decreased LC3B-II ($p < 0.05$) (Fig. 3D) compared to both untreated cell culture and culture in the presence of individual inhibitors at the corresponding concentrations.

Thus, the combination of Torin 1 and H-151 causes a multifaceted effect on autophagy, activating early stages and potentially inhibiting terminal stages, as evidenced by the accumulation of BECN1 and p62 and the reduction of LC3B-II. The manifestation of these effects depends on the cell type.

Combined Effects of the mTOR Inhibitor Torin 1 and the STING Inhibitor H-151 on Changes in CTSD and GCase Levels in Primary Culture of Peripheral Blood Macrophages from Neurologically Healthy Individuals and the SH-SY5Y Neuroblastoma Cell Line

The degree of lysosomal degradation was assessed by the levels of GCase and CTSD proteins. CTSD exists in cells in three main forms. The pro-form (pro-CTSD) matures in the Golgi apparatus; it is then processed into an enzymatically active intermediate form in endosomes (i-CTSD), transported to lysosomes, where it becomes the mature form (m-CTSD) (Fig. 4A).

In primary macrophage culture, Torin 1 (100 nM) increased pro-CTSD levels, while H-151 (0.5 μM and 1 μM) decreased them as compared to untreated cell culture (Fig. 4B). The combined action of Torin 1 and H-151 inhibitors at various concentrations decreased pro-CTSD compared to Torin 1 ($p < 0.01$), but increased it compared to H-151 at the corresponding concentrations without any change compared to untreated cell culture ($p > 0.05$) (Fig. 4B). Torin 1 (50 nM and 100 nM) decreased i-CTSD compared to cells cultivated without addition of inhibitors ($p < 0.05$), but combinations with H-151 (0.5 μM and 1 μM) increased its levels compared to the cells treated with Torin 1 alone at the corresponding concentrations (Fig. 4C). Only the combined effect of Torin 1 (50 nM) and H-151 (1 μM) increased the level of i-CTSD as compared to inhibitor-untreated cells (Fig. 4C). The level of m-CTSD decreased in response to separate exposure to the inhibitors Torin 1 and H-151 used at all concentrations studied, but increased in response to the combined effect of Torin 1 and H-151 at all combinations compared to the untreated cell culture and cells exposed to each inhibitor alone (Fig. 4D). The level of GCase increased by Torin 1 (100 nM), which we showed earlier [32], and decreased in the presence of H-151 (0.5 μM); however, the combined addition of Torin 1 and H-151 did not affect the level of GCase relative to the untreated cell culture ($p > 0.05$) (Fig. 4E). A mixture of Torin 1 (50 nM and 100 nM) and H-151 (0.5 μM) increased GCase levels compared to the addition of H-151 alone at the corresponding concentration.

In SH-SY5Y neuroblastoma cells, a combination of Torin 1 (100 nM) and H-151 (0.5 μM) increased pro-CTSD, while a combination of Torin 1 (50 nM) and H-151 (1 μM) decreased i-CTSD compared to the effect of Torin 1 alone used at the corresponding concentrations (Fig. 4B,C). All tested combinations of Torin 1 and H-151, as well as mono-treatments with Torin 1 (50 nM) and H-151 (0.5 μM and 1 μM) alone, increased m-CTSD levels compared to untreated cell cultures (Fig. 4D). Moreover, a more pronounced increase in m-CTSD was shown when SH-SY5Y cells were treated with combinations of Torin 1 (50 nM) and H-151 (0.5 μM and 1 μM) and with a combination of Torin 1 (100 nM) and H-151 (0.5 μM) as compared to mono-treatment with Torin 1 used at the corresponding concentrations (Fig. 4D). The GCase level decreased in response to treatments with all studied combinations of Torin 1 and H-151, and also in response to their mono-exposure, compared to the untreated cells ($p < 0.05$) (Fig. 4E) [32]. A more pronounced decrease was noted during the combined addition of Torin 1 (100 nM) and H-151 (0.5 μM and 1 μM) as compared to the addition of Torin 1 alone (100 nM) (Fig. 4E).

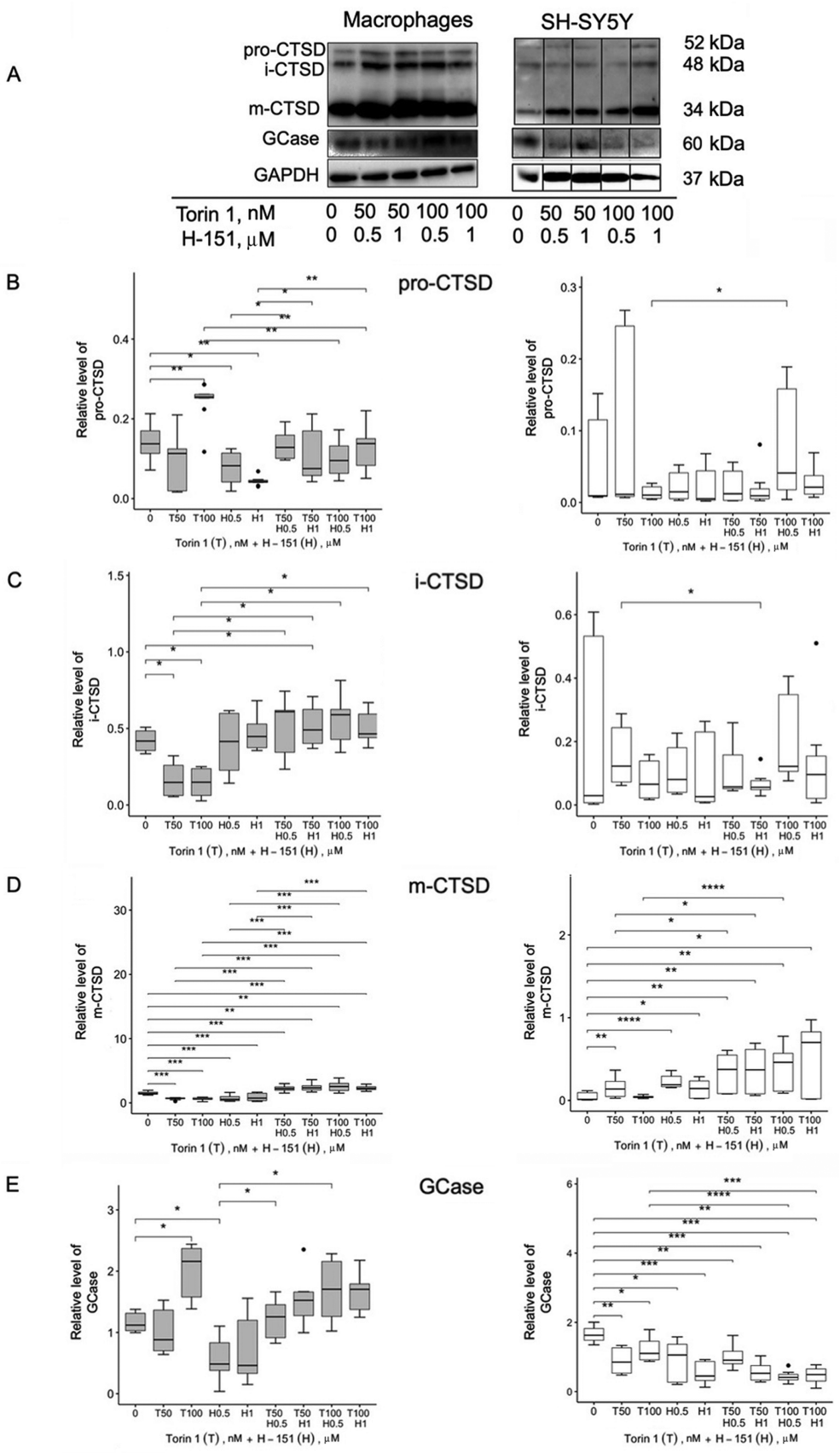

Figure 4. Evaluation of the dose-dependent combined effects of mTOR inhibition by Torin 1 and STING inhibition by H-151 in primary cultures of peripheral blood-derived macrophages (macrophages; gray bars) and SH-SY5Y neuroblastoma cells (SH-SY5Y; white bars) on lysosomal degradation. **A** – Western blotting data; **B** – relative level of pro-CTSD; **C** – relative level of i-CTSD; **D** – relative level of m-CTSD; **E** – relative level of GCase. T – Torin 1, H – H-151. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Combined Effect of the mTOR Torin 1 and the STING Inhibitor H-151 on Lysosomal Hydrolase Activity in Primary Cultures of Peripheral Blood Macrophages from Neurologically Healthy Individuals and the SH-SY5Y Neuroblastoma Cell Line

In this study we also investigated the activity of lysosomal enzymes (GALC, GCase, ASMase, GLA) and the concentration of lysosphingolipids (HexSph, LysoSM, LysoGb3) involved in ceramide metabolism and associated with the pathogenesis of PD. In primary cultures of macrophages and the SH-SY5Y neuroblastoma cells, either Torin 1 [32] and H-151 alone or in combination did not result in statistically significant changes in GALC, GCase, ASMase, and GLA activity ($p > 0.05$) compared to cells without the addition of inhibitors (Fig. 5).

At the same time, the combined treatment with Torin 1 and H-151 used at all studied concentrations decreased the concentration of HexSph in the lysates of the primary culture of peripheral blood macrophages as compared to the untreated cell culture (Fig. 6A). At all studied concentrations of Torin 1 and H-151 (except low concentrations) the combined effect of the inhibitors caused a more pronounced decrease in the HexSph concentration compared to the mono-treatments with these inhibitors used at the corresponding concentrations (Fig. 6A). The level of LysoGb3 decreased during the cultivation of the primary culture of peripheral blood macrophages in the presence of combinations of Torin 1 (50 nM and 100 nM) and H-151 (1 μM) as compared to the addition of Torin 1 alone at the corresponding concentrations (Fig. 6B). No differences were found in the concentrations of LysoSM in lysates of primary peripheral blood macrophage cultures or in the concentrations of HexSph, LysoSM, and LysoGb3 in lysates of the SH-SY5Y neuroblastoma cell line ($p > 0.05$) (Fig. 6A–C).

Combined Effect of the mTOR inhibitor Torin 1 and the STING Inhibitor H-151 on the Levels of Various Forms of Alpha-Synuclein and Tyrosine Hydroxylase in the SH-SY5Y Neuroblastoma Cell Line

In this study we investigated the combined effect of Torin 1, an mTOR protein kinase inhibitor, and H-151, a STING inhibitor, on the levels of various forms of alpha-synuclein and tyrosine hydroxylase (TH) in SH-SY5Y neuroblastoma cells (Fig. 7A).

In the SH-SY5Y cell line, their combined treatment with Torin 1 and H-151 at all concentrations studied, as well as the mono-treatment with Torin 1 (100 nM) [32] and H-151 (0.5 μM and 1 μM) resulted in a decrease in phosphorylated (Ser129)

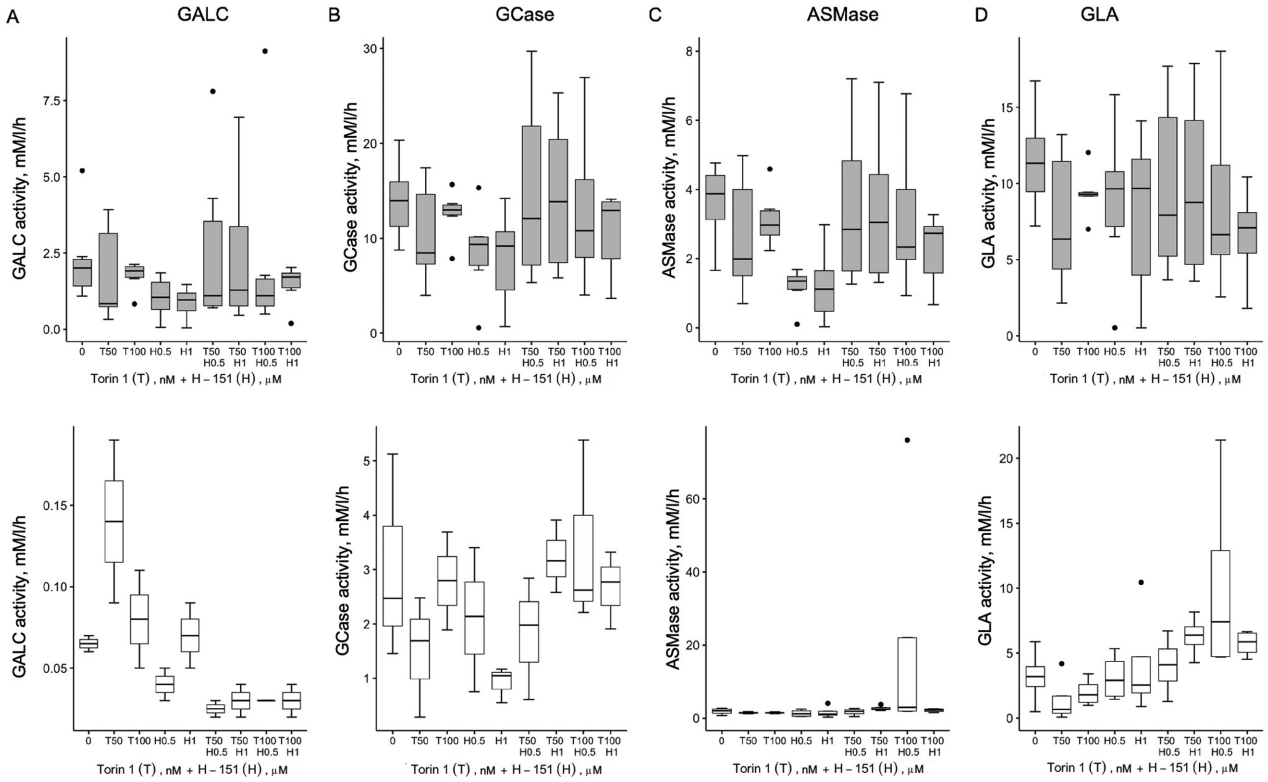

Figure 5. Evaluation of the dose-dependent combined effects of mTOR inhibition by Torin 1 and STING inhibition by H-151 in primary cultures of peripheral blood-derived macrophages (macrophages; gray bars) and SH-SY5Y neuroblastoma cells (SH-SY5Y; white bars) on the activity of lysosomal enzymes. **A** – GALC; **B** – GCase; **C** – ASMase; **D** – GLA. T – Torin 1, H – H-151.

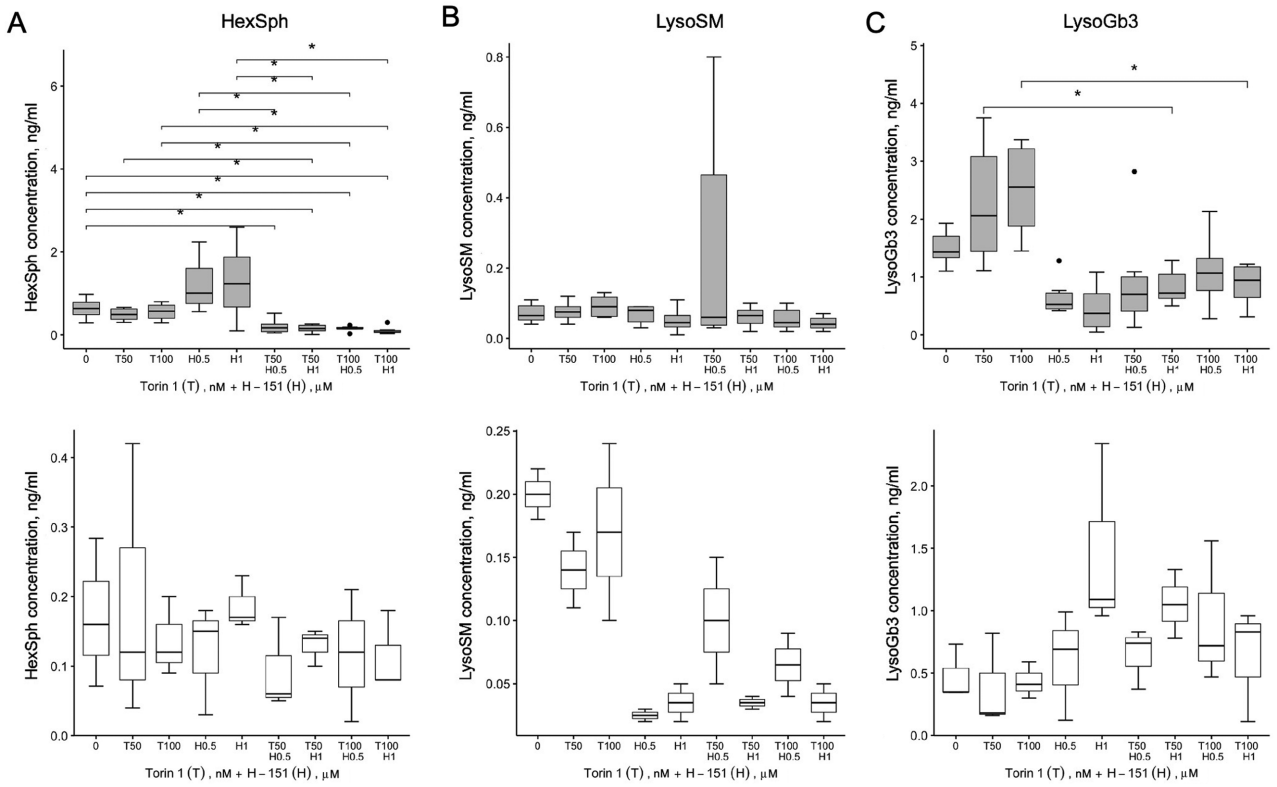

Figure 6. Evaluation of the dose-dependent combined effects of mTOR inhibition by Torin 1 and STING inhibition by H-151 in primary cultures of peripheral blood-derived macrophages (macrophages; gray bars) and SH-SY5Y neuroblastoma cells (SH-SY5Y; white bars) on lysosphingolipid levels. **A** – HexSph; **B** – LysoSM; **C** – LysoGb3. T – Torin 1, H – H-151. * $p < 0.05$.

alpha-synuclein compared to the untreated cell culture (Fig. 7B). The combinations of Torin 1 (50 nM) and H-151 (0.5 μM and 1 μM) decreased phosphorylated (Ser129) alpha-synuclein compared to Torin 1 alone at the corresponding concentration, while a mixture of Torin 1 (100 nM) and H-151 (1 μM), on the contrary, increased the protein level as compared to H-151 alone (1 μM) (Fig. 7B). A decrease in monomeric alpha-synuclein was observed during the treatment with H-151 (1 μM), Torin 1 (50 nM) [32], and H-151 (0.5 μM), Torin 1 (100 nM) and H-151 (1 μM) as compared the untreated cells (Fig. 7C). The combined effect of the inhibitors also led to a decrease in monomeric alpha-synuclein in various combinations as compared to the mono-treatments, with the only exception of a mixture of Torin 1 (50 nM) and H-151 (1 μM), which increased the level of monomeric alpha-synuclein as compared to the effect of H-151 alone (1 μM) (Fig. 7C). An increase in tetrameric alpha-synuclein was observed upon addition of Torin 1 (100 nM) to SH-SY5Y cells, both in comparison with untreated cells (as shown previously [32]) and in comparison with combinations of Torin 1 (100 nM) and H-151 (0.5 μM and 1 μM) (Fig. 7D). Interestingly, the TH level increased upon exposure to combinations of Torin 1 and H-151 at all concentrations studied as compared with untreated cell culture; a more pronounced increase was observed with the addition of a mixture of Torin 1 (100 nM) and H-151 (1 μM), and Torin 1 (50 nM) and H-151 (0.5 μM) compared to the mono-exposure of H-151 (1 μM) and Torin 1 (50 nM) ($p < 0.01$), respectively (Fig. 7E).

Measurement of alpha-synuclein protein levels in the primary culture of peripheral blood macrophages was not performed due to the low sensitivity of the method.

Dose-Dependent Effect of the mTOR Inhibitor Torin 1 and the STING Inhibitor H-151 on the Degree of Apoptosis in Primary Culture of Peripheral Blood Macrophages

The obtained results did not reveal statistically significant changes in the degree of apoptosis during cell mono-treatment with Torin 1 and H-151 inhibitors or their combination ($p > 0.05$) (Fig. 8A–D).

Additionally we investigated the synergistic effect of combined mTOR and STING inhibition on autophagy markers, the activity of lysosomal hydrolases, their substrates, various forms of alpha-synuclein, and the degree of apoptosis in primary culture of peripheral blood macrophages and SH-SY5Y cells using the HSA method

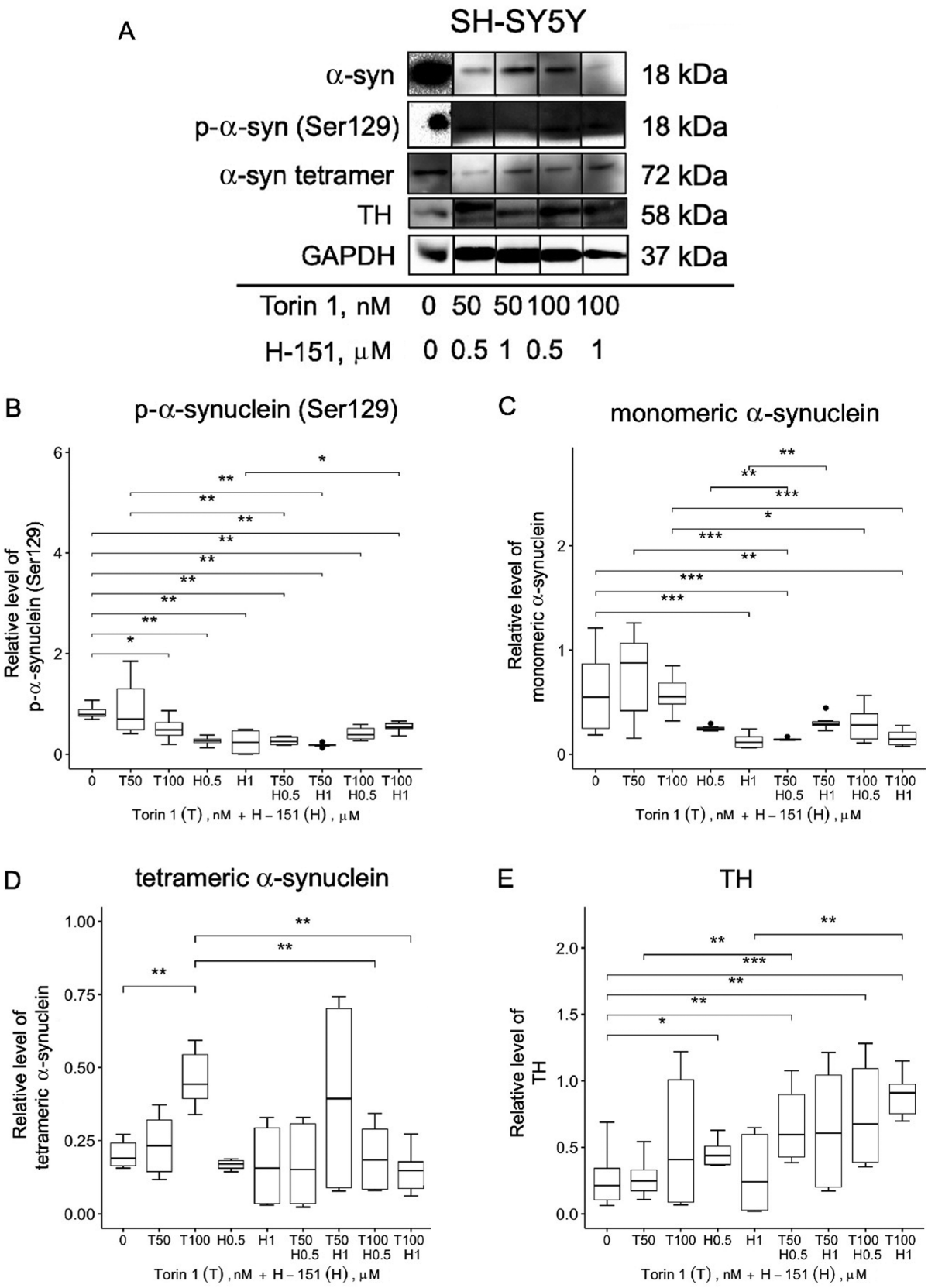

Figure 7. Evaluation of the dose-dependent combined effects of mTOR inhibition by Torin 1 and STING inhibition by H-151 in SH-SY5Y neuroblastoma cells (SH-SY5Y) on the levels of different alpha-synuclein species and tyrosine hydroxylase (TH). **A** – Western blotting data; **B** – relative level of phosphorylated alpha-synuclein (Ser129); **C** – relative level of monomeric alpha-synuclein; **D** – relative level of tetrameric alpha-synuclein; **E** – relative level of TH. T – Torin 1, H – H-151. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

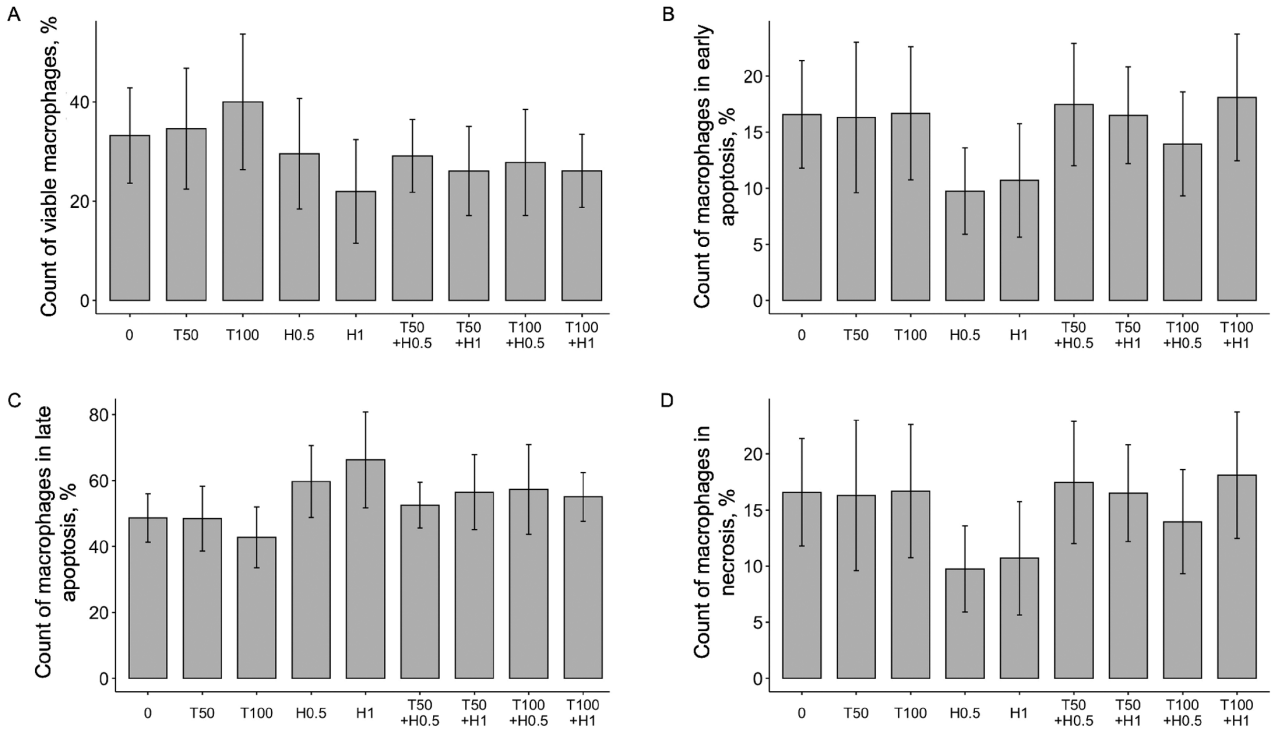

Figure 8. Evaluation of the dose-dependent combined effects of mTOR inhibition by Torin 1 and STING inhibition by H-151 in primary cultures of peripheral blood-derived macrophages on apoptosis. **A** – number of living cells; **B** – degree of early apoptosis; **C** – degree of late apoptosis; **D** – degree of necrosis. T – Torin 1, H – H-151.

for each parameter (Supplementary Materials, Tables S1, S2). The synergy index was ranked as follows: < -10 — antagonism, [-10; 5] — additivity, [5; 10] — moderate synergy, > 10 — synergy. An additive effect of combined mTOR and STING inhibition on all studied parameters was revealed in two cell lines.

DISCUSSION

In this study using primary cultures of peripheral blood macrophages from neurologically healthy donors and the SH-SY5Y neuroblastoma cell line, we have investigated for the first time the effect of combined dose-dependent inhibition of the mTOR (by Torin 1) and STING (by H-151) pathways on key parameters of the autophagolysosomal system associated with the pathogenesis of PD. It was shown that combined targeting of these signaling pathways led to activation of the autophagolysosomal system, decreased levels of the monomeric and neurotoxic phosphorylated (Ser129) forms of alpha-synuclein, along with a decrease in lysosphingolipid levels, and an increase in the mature form m-CTSD, an enzyme that plays a key role in the of alpha-synuclein degradation.

Despite significant progress in our understanding of PD pathogenesis, the molecular mechanisms still remain unclear. The accumulation of pathological forms of alpha-synuclein is considered a key event in PD. Defects in autophagy and lysosomal degradation impaired its utilization, and the resulting aggregates of this protein further disrupt mitochondrial function, leading to neuroinflammation and lysosomal dysfunction, creating a vicious cycle. In this context, autophagy and neuroinflammation are considered as central processes contributing to neuronal death and PD progression [33–37].

Protein kinase mTOR, a key component of the PI3K/AKT/mTOR signaling cascade, negatively regulates autophagy in cells. Numerous studies performed using PD and GBA1-PD models have shown that mTOR inhibitors increase the autophagic flux, stimulate lysosomal biogenesis, and reduce the levels of pathological forms of alpha-synuclein [21, 32, 38–43]. Disruption of the PI3K/AKT/mTOR signaling cascade was previously identified in our whole-transcriptome analysis of primary cultured peripheral blood macrophages from patients with GBA1-PD and *substantia nigra* from a mouse model of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced parkinsonism and conduritol-beta-epoxide-induced GCase dysfunction [20, 44]. Our data are also consistent with the results of a post-translational proteome study conducted on neurons differentiated from induced pluripotent stem cells (iPSCs) obtained from patients with GBA1-PD; this study revealed an increase in p-mTOR and other proteins associated with lysosome function [22].

In the present study, treatment of primary cultures of peripheral blood macrophages with Torin 1 was accompanied by a decrease in BECN1 and p62 levels and an increase in the lipidated form of LC3B-II. This reflects the autophagy activation and confirms data from other studies on the effect of Torin 1 on the key autophagy proteins LC3B and p62 in neurons differentiated from iPSCs of patients with GBA1-PD and in primary cultures of mouse cortical astrocytes [21, 33]. In SH-SY5Y neuroblastoma cells, Torin 1 also increased LC3B-II and caused a decrease in phosphorylated alpha-synuclein (Ser129) under conditions of increased tetrameric form of this protein [32]; however, the effect on BECN1 and p62 was less pronounced.

STING is a central mediator of the innate immune response and one of the main activators of neuroinflammation [45]. This protein, interacting predominantly with mitochondrial DNA in the cell cytoplasm, changes its conformation and acquires the ability to bind to TBK1 kinase, which phosphorylates and activates type I interferon transcription factors. A recent study has shown that STING-induced autophagy precedes TBK1 activation and type I interferon secretion; this allows us to consider autophagy as the original function of STING during evolution [46]. Moreover, STING activation can induce LC3B lipidation independently of ULK1, VPS34, and BECN1 (classical proteins that activate LC3B-II formation). STING inhibitors are currently considered as promising therapeutic strategies for diseases associated with hyperactivation of the innate immune response, including autoimmune syndromes (e.g., SAVI syndrome (STING-associated vasculopathy with early onset), systemic lupus erythematosus) [47, 48], and neurodegenerative diseases, where chronic activation of cGAS (Cyclic GMP-AMP synthase) by STING promotes neuroinflammation and may enhance alpha-synuclein-associated pathology (including PD) [49–51]. In animal models of GCase dysfunction, small molecule STING inhibitors, including H-151, have been shown to reduce STING activity, neuroinflammation, and neurodegeneration of cortical neurons [52].

In the present study, pharmacological inhibition of STING by H-151 did not affect the levels of BECN1 and p62 in primary cultured peripheral blood macrophages and the SH-SY5Y cells, but caused a decrease in LC3B-II in primary cultured peripheral blood macrophages and a tendency to decrease in the SH-SY5Y cell line. These data suggest that STING predominantly controls the stage of autophagosome formation rather than the expression of regulatory proteins; this is consistent with previous results on the autophagy regulation by STING [53, 54]. It has previously been shown that pharmacological inhibition of STING or knockout of its gene reduced inflammation, the level of phosphorylated alpha-synuclein (Ser129), and the severity of neurodegeneration in PD models *in vivo* [49, 55]. This was also found in our *in vitro* study, namely, a decrease in monomeric and phosphorylated (Ser129) alpha-synuclein in SH-SY5Y cells exposed to the H-151 inhibitor.

It is important to emphasize that there is a functional relationship between autophagy and neuroinflammation. Disruption of the autophagic flux leads to the accumulation of damaged mitochondria and the release of mitochondrial DNA, which activates the cGAS-STING pathway and secondarily triggers the NLRP3 inflammasome [56]. This is accompanied by increased secretion of proinflammatory cytokines, which has been shown in cellular and animal models of PD and is associated with motor and cognitive impairment [57]. Moreover, tumor necrosis factor α (TNF-α), characterized by increased expression in response to STING activation, is able to suppress the autophagic flux in microglia and neurons through mTOR activation, which is accompanied by overexpression of lysosomal markers LAMP1 and LAMP2 [58]. Crosstalk between STING and mTOR signaling is also realized through transcription factors of the MIT/TFE family, which regulate lysosome biogenesis and autophagy [59]. Under normal conditions, phosphorylation of MIT/TFE by mTOR kinase retains them in the cytoplasm, whereas acute STING activation promotes their dephosphorylation and translocation to the nucleus; this leads to the expression of autophagolysosomal pathway genes and activation of autophagy [46, 59]. However, in PD and other neurodegenerative diseases, chronic STING activation is accompanied by persistent secretion of proinflammatory cytokines, which suppress the autophagic flux and exacerbate lysosomal dysfunction through mTOR activation [60]. Thus, STING plays a dual role. In an acute response, it can temporarily activate autophagy, but during long-term activation, it becomes a factor of autophagy impairment and maintenance of chronic inflammation. Taken together, these data suggest that mTOR and STING should be considered as key nodes in the regulation of cellular homeostasis and promising therapeutic targets in PD. It is interesting to emphasize that TBK1 can directly activate mTOR [61], and mTOR inhibition can reduce STING levels and the production of type I interferons in peripheral blood mononuclear cells of patients with systemic lupus erythematosus [62]. In our study, inhibition of H-151 reduced p-mTOR (Ser2448), while treatment with Torin 1 reduced p-TBK1 (Ser172) in primary cultured peripheral blood macrophages, further highlighting the crosstalk between mTOR and STING signaling cascades.

Thus, simultaneous modulation of the two molecular pathways, described above, may be a promising

strategy for the treatment of sPD and GBA1-PD, as supported by clinical trials NCT06612593 and NCT04127578. A recently initiated clinical trial to reposition cilostazol for patients with intermittent claudication for the treatment of PD (NCT06612593) confirms the potential of this multiplex therapy strategy. Cilostazol has been shown to inhibit activation of the STING signaling pathway by preventing its translocation from the endoplasmic reticulum to the Golgi apparatus, which is comparable in efficacy to the selective STING inhibitor H-151 [63]. With regard to the regulation of the mTOR pathway, cilostazol has been shown to activate the SIRT1 → LKB1 → AMPK cascade; this leads to mTOR inhibition, restoration of the autophagic flux, and degradation of pathological proteins [64]. Another clinical trial (NCT04127578) of the treatment of patients with GBA1-PD proposes the use of a combination of adeno-associated virus carrying a normal copy of the *GBA1* gene with methylprednisolone, a corticosteroid that prevents the release of substances causing inflammation, and with sirolimus (rapamycin), a canonical mTOR kinase inhibitor.

In this study, we assessed the effects of simultaneous inhibition of STING and mTOR activity with small molecules on autophagolysosomal parameters and the levels of various forms of alpha-synuclein protein in primary human peripheral blood macrophage cultures and the SH-SY5Y neuroblastoma cell line. The simultaneous use of Torin 1 and H-151 resulted in activation of early stages of autophagy in both primary peripheral blood macrophages and the SH-SY5Y cells. This effect was manifested by an increase in BECN1 and accumulation of p62 with a decrease in LC3B-II, both as compared to untreated cell cultures and to single inhibitor treatment, thus indicating a possible delay in the autophagosome maturation stage. Our results are consistent with previously published data indicating that mTOR inhibition activates the initiating stages of autophagy, while inhibition of the STING pathway can affect the maturation and degradation of autophagosomes. The effect of the Torin 1 and H-151 combination is determined by both the cell type and the initial state of the autophagic system, highlighting the importance of considering the cellular context during interpretation of results and development of therapeutic strategies aimed at autophagy modulation.

In this study, we assessed the effect of mTOR (Torin 1) and STING (H-151) inhibition, as well as their combined effects, on the activity of lysosomal hydrolases (GALC, GCase, ASMase, GLA) and the levels of their substrates (HexSph, LysoSM, LysoGb3). Elevated levels of these lysosphingolipids are observed in lysosomal storage diseases (LSDs; Krabbe disease, Gaucher disease, Niemann-Pick disease type A/B, Fabry disease). Disruptions in mTOR and STING signaling have been demonstrated not only in PD but also in LSDs, where hyperactivation of mTOR substrates (p-RPS6) and STING-associated proteins (p-TBK1) [21, 38, 65], as well as impaired lysosomal degradation, chronic inflammation, and neurodegeneration were also observed [66–69].

In primary cultures of peripheral blood macrophages, combined inhibition restored lysosomal function and normalized lipid metabolism, as evidenced by a decrease in HexSph concentrations and a tendency toward a decrease in LysoGb3 as compared to monotherapy and without treatment, thus confirming the synergistic effect of mTOR and STING in regulating sphingolipid metabolism. Considering that lipid accumulation and changes in the lipid composition of membranes can induce conformational rearrangements of the alpha-synuclein protein, promoting its lipid-induced oligomerization and amyloidogenesis, the restoration of lysosomal function can limit these pathological processes associated with the pathogenesis of PD [69]. The obtained results are consistent with data on the role of mTOR in the regulation of lysosomal function [70]. They also confirm the influence of STING not only on inflammatory processes and autophagy, but also on lipid metabolism [71–73]. It is important to note the reverse effect: activation of the STING pathway was shown in a primary culture of mouse glial cells with GCase dysfunction and lysosphingolipid accumulation [52]. Considering the role of lipids in the initiation of alpha-synuclein aggregation [74–78], their reduction could serve as an important neuroprotective mechanism.

Of particular significance in our study is the detected increase in the mature form m-CTSD observed during combined inhibition of mTOR and STING as compared to single inhibitor treatment and without added inhibitors. CTSD has been chosen as a marker of lysosomal activity because this enzyme is widely expressed in the brain and plays a key role in maintaining lysosomal-dependent protein homeostasis, including the degradation of pathological forms of alpha-synuclein [79, 80]. In PD models, its haploinsufficiency leads to lysosomal dysfunction and intercellular transfer of alpha-synuclein aggregates [81]. Our data show that an increase in the level of mature m-CTSD in primary cultures of peripheral blood macrophages and the SH-SY5Y cells treated with combination of mTOR and H-151 can be considered as a positive marker of lysosomal function restoration. Moreover, a decrease in the levels of both monomeric and serine 129-phosphorylated alpha-synuclein was observed in neuroblastoma, thus indicating the effectiveness of combined mTOR and STING inhibition in reducing pathogenic forms of the protein.

Interestingly, combined use of the inhibitors was not pro-apoptotic in primary cultures of peripheral blood macrophages, suggesting the relative safety of this strategy [82–85].

CONCLUSIONS

This study has shown that the simultaneous use of mTOR (Torin 1) and STING (H-151) inhibitors influences autophagy and related processes. In SH-SY5Y cells, the combined treatment resulted in activation of early stages of native autophagy, manifested by increased BECN1 and p62 levels and a decrease in LC3B-II, which may indicate the initiation of the autophagic process with a possible delay in the autophagosome maturation stage. Simultaneously, increased lysosomal degradation was observed, as evidenced by an increase in the mature form m-CTSD. In primary cultures of peripheral blood macrophages, combined use of Torin 1 and H-151 also resulted in a decrease in LC3B-II without significant changes in BECN1 and p62 levels, which may reflect the activation of early stages of autophagy and the normalization of autophagic flux. Furthermore, combined inhibition had a normalizing effect on lipid metabolism, which was also confirmed by an increase in the mature form m-CTSD and a decrease in the sphingolipid HexSph, and was not accompanied by activation of apoptosis, indicating the preservation of cellular viability with the simultaneous action of Torin 1 and H-151. These results highlight differences in the response of cells exposed to the same small molecules; this is consistent with previously published data obtained in PD modeling in various cellular models *in vitro*, including neuron-like cell lines (SH-SY5Y, LUHMES), primary neurons, glial cells, and microglia [86–88].

Thus, our results demonstrate that simultaneous targeting of the mTOR and STING signaling pathways simultaneously affects multiple links in the pathological cascade, including autophagy, sphingolipid metabolism, and the reduction of monomeric alpha-synuclein and its potentially neurotoxic Ser129-phosphorylated forms. These data confirm the potential of this approach for correcting impairments characteristic of neurodegenerative diseases, particularly PD. Further development of this approach requires additional studies using patient-specific *in vitro* cell models and animal models to evaluate the efficacy and safety of combined inhibition in pathology and at the organismal level.

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COMPLIANCE WITH ETHICAL STANDARDS

Peripheral blood samples for this study were obtained in accordance with the principles of the Declaration of Helsinki and current ethical standards. All volunteers signed written informed consent to participate in the study. The study was approved by the Ethics Committee of the First Pavlov State Medical University of St. Petersburg (Protocol no. 275, dated September 4, 2023).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

Supplementary materials are available in the electronic version at the journal site (pbmc.ibmc.msk.ru).

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ОДНОВРЕМЕННОЕ ИНГИБИРОВАНИЕ mTOR И STING КАК ПОДХОД К СНИЖЕНИЮ УРОВНЯ АЛЬФА-СИНУКЛЕИНА И ЛИЗОСФИНГОЛИПИДОВ В ПЕРВИЧНОЙ КУЛЬТУРЕ МАКРОФАГОВ ПЕРИФЕРИЧЕСКОЙ КРОВИ ЧЕЛОВЕКА И КЛЕТОЧНОЙ ЛИНИИ НЕЙРОБЛАСТОМЫ SH-SY5Y: ПЕРСПЕКТИВЫ ТЕРАПИИ БОЛЕЗНИ ПАРКИНСОНА

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В первичной культуре макрофагов периферической крови здоровых доноров и клеточной линии нейробластомы SH-SY5Y исследовали сочетанное влияние разнонаправленных препаратов: ингибитора mTOR (Torin 1), ключевого регулятора аутофагии, и ингибитора STING (H-151), ключевого регулятора воспаления, на параметры аутофаголизосомной системы. Сочетанное применение этих препаратов приводило к снижению уровня лизосфинголипидов, являющихся триггерами олигомеризации альфа-синуклеина, а также к снижению уровня мономерного и нейротоксичного фосфорилированного (Ser129) альфа-синуклеина и увеличению уровня тирозингидроксилазы. Полученные результаты открывают новые перспективы для применения комбинированной терапии предложенных препаратов в лечения как заболеваний, связанных с дисфункцией лизосом, так и с нейродегенеративными патологиями.

Полный текст статьи на русском языке доступен на сайте журнала (http://pbmc.ibmc.msk.ru).

Ключевые слова: болезнь Паркинсона; mTOR; STING; альфа-синуклеин; активность лизосомных ферментов; аутофагия

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