

# Inhibition of STAT Transcription Factor Attenuates MPP<sup>+</sup>-induced Neurotoxicity

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**Background:** The most prominent pathological features of Parkinson's disease (PD) are diminished substantia nigra (SN), which is part of the output component of the basal ganglia, the severe death of dopaminergic neuronal cell and the accumulation of a synuclein ( $\alpha$ SYN). However, the mechanism by which  $\alpha$ SYN causes toxicity and contributes to neuronal death remains unclear.

**Methods:** The aim of this study was to investigate the effect of  $\alpha$ SYN/STAT oligodeoxynucleotide (ODN), which simultaneously suppresses STAT transcription factors and  $\alpha$ SYN mRNA expression in an *in vitro* Parkinson's disease model.

**Results:** Synthetic  $\alpha$ SYN/STAT ODN effectively inhibits 1-Methyl-4-phenylpyridinium (MPP<sup>+</sup>) induced STAT phosphorylation and  $\alpha$ SYN expression.  $\alpha$ SYN/STAT ODN attenuated MPP<sup>+</sup> to mimic PD model *in vitro*. MPP<sup>+</sup> induced the secretion of TNF- $\alpha$ /IL-6, inhibited cell viability and induced apoptosis while these effects could be rescued by  $\alpha$ SYN/STAT ODN.

**Conclusion:** Therefore, synthetic  $\alpha$ SYN/STAT ODN has substantial therapeutic feasibility for the treatment of neurodegenerative diseases.

**Key words:** Parkinson's disease, Oligodeoxynucleotide,  $\alpha$ -Synuclein, Apoptosis, STAT

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## INTRODUCTION

Parkinson's disease (PD) is a chronic, widespread neurodegenerative disorder characterized by a progressive loss of dopaminergic neurons in the substantia nigra (SN) pars compacta (SNpc) throughout the midbrain [1]. The main pathology of PD is the aggregation of the protein  $\alpha$ -synuclein ( $\alpha$ SYN) in the cytoplasmic region of dopamine neurons [2].

The Janus Kinase/Signal Transducers and Activators of Transcription (JAK/STAT) is activated by cytokines, interferons, and growth factors [3] and is involved in cell survival, proliferation, angiogenesis, inflammation, and apoptosis [4]. Abnormal activation of JAK/STAT occurs in neuroinflammation and neurodegenerative diseases such as multiple sclerosis, Alzheimer's disease, and PD [5].

Synthetic oligodeoxynucleotide (ODN) technology is a gene therapy strategy consisting of DNA or RNA-based molecular compounds that disrupt gene transcription or translation [6]. To improve a new therapeutic approach, in this study we used a combination of antisense ODN and decoy ODN to synthesize  $\alpha$ SYN/STAT ODN which inhibits both SYN and STAT. Although these ODN have proven beneficial in several disease models, it has not yet been demonstrated whether  $\alpha$ SYN/STAT ODN can attenuate the development of molecular mechanisms of neurotoxicity. Therefore, we investigated the effect of  $\alpha$ SYN/STAT ODN on neuronal cytotoxicity in an *in vitro* model of Parkinson's disease.

## METHODS

### Synthesis of Oligodeoxynucleotides (ODN)

Synthetic ODNs were commissioned by Macrogen (Seoul, Korea). The synthetic decoy ODN sequences were used as follows (the target site of the consensus sequence is underlined): STAT decoy ODN: 5' GAA TTC GTT TCC GGG AAT GAA AAC ATT CCC GGA AAC 3';  $\alpha$ SYN antisense ODN: 5' GGT ACC CTT CTT CAC CCT TAC C 3'; scrambled (SCR) decoy ODN: 5' GAA TTC AAT TCA GGG TAC GGC AAA AAA TTG CCG TAC CCT GAA TT 3'. Considering the stability of the decoy ODN strategy, we designed a ring-type structured decoy ODN. These ODNs were annealed for 6 hours while temperature was gradually decreased from 80°C to 25°C. Each ODN was mixed with T4 ligase (Takara Bio, Otsu, Japan) and incubated for 18 hours at 16°C to obtain a covalent ligation for the ring-type decoy ODNs.

### Cell culture and Reagents

A dopaminergic human neuroblastoma cell line SH-SY5Y (America Tissue Culture Collection, CRL-2266; ATCC, Manassas, VA, USA), was cultured in a Dulbecco's Modified Eagle's Medium (DMEM) medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Gibco) and 1% Anti-Anti (Gibco). Cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. The sources of the following reagents were: 1-Methyl-4-phenylpyridinium ion (MPP<sup>+</sup>) (Sigma-Aldrich); anti-SYN (Cat no: 2628, Cell Signaling Technology), anti-PARP-1 (Cat no: 9542, Cell Signaling Technology), anti-pSTAT3 (Cat no: 9145, Cell Signaling Technology) and anti- $\beta$ -actin (Cat no: SAB3500350, Sigma-Aldrich). Immunoblots were detected using an enhanced chemiluminescence reagent (Amer-sham Bioscience, Amersham, UK).

### Cytotoxicity assay

To evaluate the effect of  $\alpha$ SYN/STAT ODN on MPP<sup>+</sup> stimulated proliferation SH-SY5Y cells were plated in 96-well culture plates at  $1 \times 10^5$  cells/ml in culture medium and allowed to attach for 24 hours. Media were discarded and transfect with  $\alpha$ SYN/STAT ODN in a new medium, then treat with MPP<sup>+</sup> for 24 hours. Cell viability was analyzed using the Cell Counting Kit (CCK-8; Dojindo Laboratories, Kumamoto, Japan) assay according to the manufacturer's instructions. The absorbance at 450 nm was assessed using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

### Transfection and Morphology examination

SH-SY5Y cells were transfected with synthetic ODN using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After transfection, the SH-SY5Y cells were cultured in MPP<sup>+</sup> for 24 hours. The morphology of SH-SY5Y cells were observed using an inverted phase contrast microscope (Olympus CKX41SE, Tokyo, Japan,  $\times 200$  magnification).

### Immunoblot analysis

SH-SY5Y cells with a protein extraction buffer (N-PER<sup>™</sup>, Thermo Fisher Scientific, Waltham, MA, USA) according to the instruction manual. The protein samples were separated on precast gradient polyacrylamide gels (Bolt<sup>™</sup> 4–12% Bis-Tris Plus Gels; Thermo Fisher Scientific) and transferred to nitrocellulose membranes (GE Healthcare, Madison, WI, USA) by using Bolt<sup>™</sup> Mini Blot Module and Mini Gel Tank (Thermo Fisher Scientific), according to the manufacturer's recommendations. The membrane blocked with 5% bovine serum albumin was probed with a primary antibody and horseradish peroxidase-conjugated secondary antibody. Following a repeat of the wash step, the membrane was kept in enhanced chemiluminescence detection reagents (Thermo Fisher Scientific). Signal intensity was measured with an image analyzer (Chemidoc<sup>™</sup> XRS+; Bio-Rad Laboratories).

### Enzyme-linked immunosorbent assay (ELISA)

The culture medium of the cells was harvested, and cytokine production (TNF $\alpha$  and IL6) in the supernatant was measured with a solid phase sandwich ELISA using a Quantikine TNF $\alpha$  and IL6 kit (R&D systems, MN, USA) according to the manufacturer's instructions.

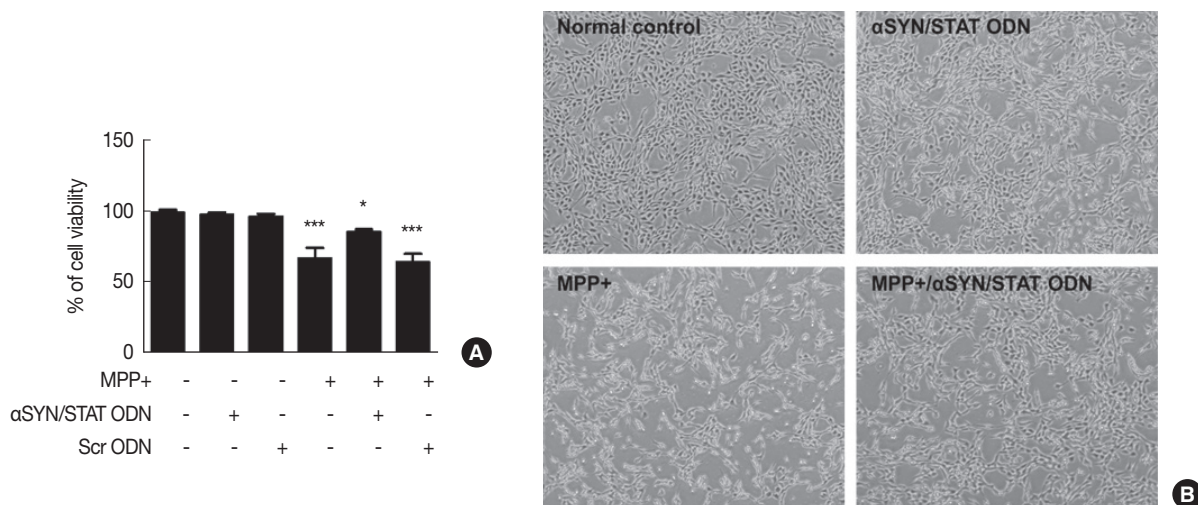
### Statistical analysis

All data analysis was performed with the GraphPad Prism 9 (GraphPad Software, Inc., San Diego, CA, USA) using either a one-way ANOVA with Tukey's post hoc test for multiple comparisons and data are presented as the mean  $\pm$  SEM (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).

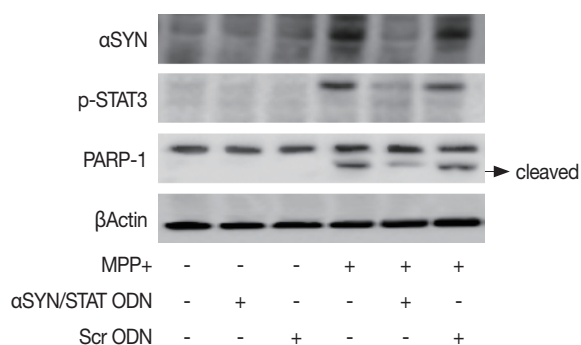
## RESULTS

### $\alpha$ SYN/STAT ODN protects SH-SY5Y cells against MPP<sup>+</sup> induced neurotoxicity

The cytotoxic effects of  $\alpha$ SYN/STAT ODN on SH-SY5Y cells were examined through a CCK assay before investigating its



**Fig. 1.** Effect of  $\alpha$ SYN/STAT oligodeoxynucleotide (ODN) on MPP<sup>+</sup> to mimic Parkinson's disease (PD) model *in vitro*. (A) Viability was determined using the MTT assay. (B) The morphological changes, magnifications  $\times 200$ . The data are representative of three similar experiments and quantified as mean values  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared to normal control.

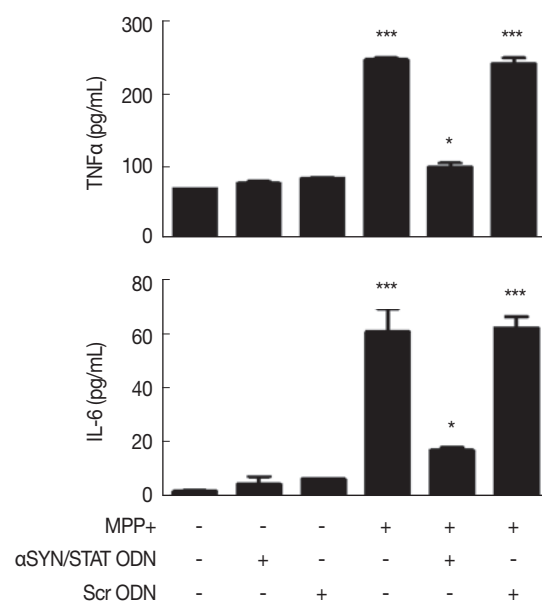


**Fig. 2.** Effect of  $\alpha$ SYN/STAT oligodeoxynucleotide (ODN) on MPP<sup>+</sup> induced SYH accumulation, STAT3 phosphorylation and cleaved PARP-1. Beta-actin was used to confirm equal sample loading.

pharmacological potential.  $\alpha$ SYN/STAT ODN significantly increased the viability of 3 mM MPP<sup>+</sup> stimulated SH-SY5Y cells compared to cells treated with only MPP<sup>+</sup> (Fig. 1A). Transfection of Scr ODN, a negative control, was similar to cells treated with MPP<sup>+</sup>. These results were also observed in cell morphology. SH-SY5Y cells grew well, showing obvious neurites, and the cells treated with only  $\alpha$ SYN/STAT ODN did not show any difference in cell growth compared to normal cells (Fig. 1B). When SH-SY5Y cells were exposed to MPP<sup>+</sup> or Scr ODN, neurites were reduced and cell debris increased; however, they were recovered with  $\alpha$ SYN/STAT ODN transfection.

#### Effect of $\alpha$ SYN/STAT ODN on MPP<sup>+</sup> induced apoptosis signaling pathway

Since apoptosis is one of the important steps in the patho-



**Fig. 3.** Effect of  $\alpha$ SYN/STAT oligodeoxynucleotide (ODN) on MPP<sup>+</sup> induced neuroinflammatory responses. The data are representative of three similar experiments and quantified as mean values  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared to normal control.

genesis of PD, we hypothesized that  $\alpha$ SYN/STAT ODN could protect dopaminergic neuronal cells by inhibiting the apoptotic pathway. First, we confirmed changes in the expression of ODN target proteins caused by MPP<sup>+</sup>. As shown Fig. 2, expression of SYN and p-STAT3 were increased by MPP<sup>+</sup> or Scr ODN. As expected, this increase was reduced by  $\alpha$ SYN/STAT ODN. In addition,  $\alpha$ SYN/STAT ODN inhibited MPP<sup>+</sup> induced cleaved PARP-1, apoptosis marker protein, in SH-SY5Y cells.

## $\alpha$ SYN/STAT ODN alleviates MPP<sup>+</sup> induced neuroinflammatory response

MPP<sup>+</sup> causes mitochondrial dysfunction and neuroinflammation [7]. Repression of the JAK/STAT pathway disrupts the neuroinflammation and neurodegeneration circuitry characteristic of PD [8]. To evaluate the impact of  $\alpha$ SYN/STAT ODN on MPP<sup>+</sup> mediated neuroinflammatory response, SH-SY5Y cells were transfected with  $\alpha$ SYN/STAT ODN and Scr ODN followed by MPP<sup>+</sup> for 24 hours. The secretion of TNF $\alpha$  and IL6 were significantly inhibited in MPP<sup>+</sup> stimulated SH-SY5Y cells by  $\alpha$ SYN/STAT ODN transfection (Fig. 3). Scr ODN was similar to cells treated with MPP<sup>+</sup>.

## DISCUSSION

The first peptide inhibitors of STAT proteins were discovered more than a decade ago, and attempts to target STAT signaling for therapeutic purposes are still ongoing [9]. Aberrant activation of the JAK/STAT pathway contributes to a number of autoimmune and neuroinflammatory diseases [10]. Several studies have illustrated that the novel inflammatory signals namely JAK/STAT, can be activated by LPS, TNF- $\alpha$ , IFN- $\gamma$ , and IL-6 in the brain [11] and contribute to the pathogenesis of neuroinflammatory diseases [5]. The  $\alpha$ SYN accumulation in the brain activated microglial and produced inflammatory cytokines or chemokines through the activation of the JAK/STAT pathway in different models of PD [12]. In addition, neurotoxin MPP<sup>+</sup> treatment increased STAT1 expression levels and STAT1 phosphorylation and subsequent apoptosis in cerebellar granule neuron cells [13]. Furthermore, pyridone 6, a JAK inhibitor, reduced interferon  $\beta$  neurotoxicity in SH-SY5Y cells by reducing STAT1 and STAT3 phosphorylation and apoptosis [14].

Our research investigated the  $\alpha$ SYN/STAT ODN protective effects on neurotoxicity in SH-SY5Y cells treated by MPP<sup>+</sup>. Our results exhibited that MPP<sup>+</sup> exposure induced neuroinflammatory responses and apoptosis through the secretion of TNF $\alpha$ /IL6 and expression of cleaved PARP-1 in SH-SY5Y cells. In contrast, the transfected  $\alpha$ SYN/STAT ODN reversed these changes caused by MPP<sup>+</sup> in SH-SY5Y cells. These results strongly support the effectiveness of  $\alpha$ SYN/STAT ODN, as the effect of Scr ODN was not observed.

Thus, gene therapy targeted to suppress mRNA level of SYN and transcription activity of STAT simultaneously might provide a new therapeutic strategy to prevent various neurological disorders.

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## CONFLICTS OF INTEREST

Not applicable.

## AUTHOR CONTRIBUTIONS

Investigation: Park J, Jang KM. Writing—original draft preparation: Park J, Jang KM. Writing—review: Jang KM. Funding acquisition: Park J. All authors have read and agreed to the published version of the manuscript.

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