Parkinson Disease-Modification Encompassing Rotenone and 6-Hydroxydopamine Neurotoxicity by the Microtubule-Protecting Drug Candidate SKIP

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Abstract

Encompassing live cell imaging and morphometrics at the microscopical level, we showed here, for the first time, protection of neuronal-like cells by the novel drug candidate, SKIP, against the Parkinson's disease-related neurotoxin, rotenone. Mechanistically, rotenone disrupted microtubule dynamics, which SKIP partially repaired through microtubule end-binding proteins, coupled with increasing neurite branch length. Given the previous association of rotenone toxicity with increased dopaminergic cell death hallmarking Parkinson's disease, we chose an established rat model of 6-hydroxydopamine (6-OHDA) toxicity to initially evaluate SKIP in vivo. SKIP pretreatment showed protection against nigral dopaminergic cell degeneration and improved motor behavior in the forelimb asymmetry test. With Parkinson's disease being a major neurodegenerative disorder, afflicting millions of people globally, and with disease modification challenges, SKIP may hold promise for future therapeutic development.

Introduction

Neurodegenerative diseases in general and Parkinson disease (PD) in particular are posing a significant challenge to public health. Here, we focus on intervention and prevention or modification of the disease process by targeting fundamental brain protection. Twenty years ago, the Gozes laboratory discovered a new protein and named it activity-dependent neuroprotective protein (ADNP) (Bassan et al. 1999). ADNP (Zamostiano et al. 2001) has transcription

factor properties regulating > 400 genes during development (Mandel et al. 2007) and controls microtubules (MTs). As such, ADNP is essential for brain formation and function (Pinhasov et al., 2003; Amram et al. 2016). ADNP contains a *NAPVSIPQ* (NAP) domain, including the SxIP motif, which interacts with MT end-binding proteins (EB1, EB3) enhancing/replacing ADNP-MT interactions, thereby regulating axonal transport and synapse formation (Oz et al. 2014; Amram et al., 2016; Hacohen-Kleiman et al. 2018). ADNP deficiency in mice results in vocalization, cognitive, and motor impediments and tau pathology (Vulih-Shultzman et al. 2007; Hacohen-Kleiman et al. 2018). NAP treatment corrects these abnormalities (Vulih-Shultzman et al. 2007; Hacohen-Kleiman et al. 2018; Sragovich et al. 2019). Mechanistically, NAP enhances dynamic tau interactions with MTs through MT end-binding proteins (Ivashko-Pachima et al. 2017; 2021a; 2021b). The Gozes laboratory recently designed SKIP (based on the SxIP MT-interacting motif in NAP). SKIP interacts with the NAP motif in ADNP to protect MTs and provides neuroprotection in the *Adnp*-deficient mouse, which mimics the autistic/intellectual disability-associated ADNP syndrome (Amram et al. 2016; Hacohen-Kleiman et al. 2018; Ivashko-Pachima and Gozes 2019a).

A potentially important link between ADNP and PD, a neurodegenerative disease rather than a disease of neuronal development (like the ADNP syndrome), is that brain tissue

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from PD patients exhibits markedly reduced ADNP protein levels in neuromelanin-containing nigral neurons. Reduced ADNP levels occur early in PD (Chu et al. 2016) before reductions in catecholaminergic innervation (as indicated by tyrosine hydroxylase) are detected. Interestingly, our most recent findings (Hadar et al. 2021) identified two key interaction sites for ADNP and sirtuin 1 (SIRT1, a protein positively associated with aging, that in turn is linked with PD). One site is at the EB1 and EB3/tau level, with EB1/EB3 serving as amplifiers for MT dynamics, synapse formation, axonal transport and protection against tauopathy. The second is on the DNA/chromatin site, with yin yang 1 (YY1), histone deacetylase 2 (HDAC2) and ADNP sharing a DNA-binding motif and regulating SIRT1, ADNP and EB1 (also known as MAPRE1). This interaction was linked to sex- and age-dependent altered histone modification, associated with ADNP/SIRT1/WD repeat-containing protein 5 (WDR5), that mediates the assembly of histone modification complexes. In postmortem brains, we demonstrated a tight correlation for the gene transcripts described above, including related gene products. When analyzing PD brains in comparison to controls, the correlation was maintained except for the most affected substantia nigra, suggesting an important role for ADNP in PD (Hadar et al. 2021).

Furthermore, ADNP levels are also decreased in a rat model of PD (Chu et al. 2016) based on viral over-expression of human wild-type α -synuclein (AS, the major risk gene for PD), establishing a potential association between ADNP and AS-PD. Thus, downregulation of ADNP might contribute to dopaminergic neurodegeneration via AS in PD. Complementing these findings, in vitro NAP protects against (1) dopamine (DA) and 6-hydroxydopamine (6-OHDA) toxicity in rat pheochromocytoma PC12 cells and human neuroblastoma cell lines (an established model for PD) (Offen et al. 2000), (2) AS oligomerization/aggregation (Melo et al. 2017), (3) PD mitochondria-inhibited transport, (4) PD-associated MT dysfunction, and (5) reduced autophagic flux (Esteves et al. 2014). NAP also protects against MT dysfunction/tauopathy in an AS PD mouse model (Fleming et al. 2011; Magen et al. 2014). Compared to NAP, SKIP offers an advantage as a potential disease-modifying treatment of PD in that it is a smaller molecule consisting of only 4 amino acids and is therefore more likely to cross the blood–brain barrier in equal or potentially higher amounts (Gozes et al. 2005; Gozes 2020; Amram et al. 2016) and protect against axonal deficits as evidenced in the *Adnp*-deficient mice (Amram et al. 2016; Ivashko-Pachima and Gozes 2019a).

The movement deficits defining PD are well known to result from drastic depletion of DA in the nigrostriatal system (Kish et al. 1988). According to the catecholaldehyde hypothesis, cytoplasmic accumulation of the catecholaldehyde 3,4-dihydroxyphenylacetaldehyde (DOPAL), the

immediate product of the action of monoamine oxidase (MAO) on cytoplasmic DA, is central to the degenerative process (Burke et al. 2003). In PD, three abnormalities tend to build up cytoplasmic DOPAL: (1) decreased vesicular sequestration of cytoplasmic catecholamines, (2) decreased metabolism of DOPAL by aldehyde dehydrogenase (ALDH) (Goldstein et al. 2013), (3) decreased delivery of vesicles to the terminals mediated by MT depolymerization. Postmortem studies analyzing catechol patterns in putamen tissue from PD patients have confirmed this “triple hit.” Importantly, the extent of catecholamine depletion in PD is greater than can be accounted for by denervation alone. The implication is that there is a population of dysfunctional neurons that are “sick but not dead” and thus potentially salvageable.

In summary, PD is characterized by DAergic neuronal death in the midbrain and subsequent impairment in motor functions. Inhibition of mitochondrial complex I of the mitochondrial respiratory chain (Schapira 2008) and/or defective regulation of MTs (Choi et al. 2011) have been proposed as possible mechanisms of this selective neurodegeneration.

Rotenone is a well-known pesticide/insecticide that inhibits mitochondrial complex I and also depolymerizes MTs (Srivastava et al. 2007). Thus, combining catecholamines and MTs in PD toxicity, several studies have examined mechanisms besides complex I inhibition by which rotenone induces neurotoxicity. One of these is MT assembly (Srivastava and Panda 2007; Passmore et al. 2017). Other studies (Ren and Feng 2007) showed that rotenone is toxic to DA neurons in midbrain cultures and that the MT-stabilizing drug taxol mitigates this effect. MT depolymerization disrupts vesicular transport along the MT shaft and causes accumulation of DA vesicles in the soma. This leads to increased oxidative stress due to oxidation of cytosolic DA leaking from vesicles. Concurrently, decreased delivery of vesicles and vesicle-associated proteins to the terminals would be expected to limit vesicular sequestration of locally synthesized DA. These results suggest that MT depolymerization induced by toxins such as rotenone participate in the vulnerability of DA neurons at both the cell soma and the terminals (Ren et al. 2003, 2005; Ren and Feng 2007).

In cell culture models for measuring MT dynamics (Ivashko-Pachima et al. 2017, 2019b, 2021b), SKIP protects against MT disruption by zinc intoxication associated with Alzheimer's disease (Ivashko-Pachima and Gozes 2019a). Here, we asked whether this protection by SKIP is extended in vitro to protection against rotenone and in vivo to attenuate nigrostriatal 6-OHDA neurotoxicity. We hypothesized that SKIP protects catecholaminergic neurons and ameliorates the neurobehavioral, neurochemical, and neuropathological effects of rotenone and/or 6-OHDA toxicity.

Materials and Methods

Materials

SKIP (under patent protection, Ramot @ Tel Aviv University, I.G., inventor) was custom synthesized by Hai Laboratories.

Methods

Cell Cultures. Human neuroblastoma SH-SY5Y cells (passage numbers 14–16, ECACC, Public Health England, Porton Down, Salisbury, UK) and mouse neuroblastoma N1E-115 cells (passage numbers 16–18, ATCC, Bethesda, MD, USA) were plated onto 96-well plates at a concentration of 3000 cells/well and then differentiated during a week with retinoic acid (10 μ M) or with reduced FBS (2%) and DMSO (1.25%), respectively.

Cell Viability Assay (XTT). On the experimental day, differentiated SH-SY5Y cells were treated with 1, 10, and 100 μ M of rotenone during 24 or 48 h. Since the rotenone stock had been originally diluted into DMSO, “Control” groups were performed with equal volume of DMSO alone. Results of mitochondrial activity (cell viability) were obtained by the XTT colorimetric assay, according to the manufacturer’s instructions (XTT-based cell proliferation kit, Biological Industries, Beit Haemek, Israel). Statistical analysis was performed by one-way ANOVA, Tukey, or LSD HSD (IBM SPSS 23): $*p < 0.05$, $***p < 0.001$.

Cell Morphology and Polymerized vs. Soluble Microtubule Assay. Human neuroblastoma SH-SY5Y cells were plated onto 10-cm plates and differentiated as described above. Cells were treated with 1 μ M or 10 μ M rotenone during 2 or 24 h with or without SKIP at 10^{-15} M, 10^{-12} M, or 10^{-9} M. Cell branch (neurite) length was measured by Fiji (NIH) (Schindelin et al. 2012), and data were analyzed by one-way ANOVA, Tukey HSD (IBM SPSS 23): $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

After pictures of cell morphology had been taken, cells were harvested, and lysates were separated into polymerized (P) and soluble (S) protein fractions. Equal aliquots of each pair (P and S) were resolved on adjacent lanes by SDS polyacrylamide gel electrophoresis (Oz et al. 2014). The blots were probed with tubulin antibodies (alpha-tubulin, T6199, Sigma, Rehovot, Israel). The intensity of each band was quantified by Fiji, and the polymerized ratios were calculated by dividing the intensity value of the polymerized band over the sum of intensity values of polymerized and soluble bands [P/(S + P)]. Data were statistically analyzed by one-way ANOVA, LSD HSD (IBM SPSS 23) ($n = 2/\text{group}$): $*p < 0.05$.

Time-Lapse Live Cell Imaging of EB3 Comet-like Structures. Forty-eight hours before live imaging, differentiated N1E-115 cells were transfected with 1 μ g of EB3-RFP-expressing plasmid (Ivashko-Pachima et al. 2017). On the experimental day, differentiated N1E-115 cells were treated for 1 h with rotenone at final concentrations of 0.1, 1, and 10 μ M, with or without SKIP at 10^{-15} M, 10^{-12} M, and 10^{-9} M. During time-lapse imaging, N1E-115 cells were incubated at 37 °C with a 5% CO₂/95% air mixture in a thermostatic chamber placed on the stage of a Leica TCS SP5 confocal microscope (objective 100 (PL Apo) oil immersion, NA 1.4). Time-lapse images were automatically captured every 3 s for 1 min, using the Leica LAS AF software (Leica Microsystems, Wetzlar, Germany). Data were collected and analyzed by Imaris software (Bitplane, Concord, MA, USA). Statistical analysis was done by one-way ANOVA, Tukey HSD (IBM SPSS 23): $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

In vivo SKIP Injections and Partial Lesion 6-OHDA Rat Model. Adult male Sprague–Dawley rats (~250 g) were anesthetized and placed into a stereotaxic frame. SKIP (5 pg or 500 pg) or saline vehicle in a volume of 2 μ l diluted in saline was administered intraparenchymally (supranigral injection) in a single injection via a Hamilton syringe (AP – 5.2 mm, ML – 1.8, DV – 7.2; coordinates according to the rat brain atlas of Paxinos and Watson (1998)). Two weeks following the supranigral SKIP injections, the rats were re-anesthetized, placed into a stereotaxic apparatus, and received two unilateral injections of the catecholamine-specific neurotoxin 6-OHDA (10 μ g each in 2 μ l saline + 0.2% ascorbic acid) placed into the right striatum to create the partial lesion model (AP + 1.6 mm, ML – 2.4, DV – 4.2, and AP + 0.2, ML – 2.6, DV – 7.0 (Paxinos and Watson, 1998)). The striatal injection of 6-OHDA results in a relatively progressive degeneration of the DAergic nigrostriatal pathway (Sauer and Oertel, 1994; Kirik et al. 1998; Deumens et al. 2002). All animal experiments were performed following an approved protocol by the University of Cincinnati Institutional Animal Care and Use Committee.

Forelimb Asymmetry (“Cylinder”) Test. Animals were tested for motor deficits using the forelimb-use asymmetry test, as described previously (Schallert et al. 2000; Hemmerle et al. 2014; Hu et al. 2017; Kyser et al. 2019). This behavioral test is a simple, noninvasive test of a naturally occurring behavior that is particularly useful in unilateral partial lesion models (Schallert et al. 2000; Lundblad et al. 2005). The procedure was performed under video surveillance and at least an hour into the dark cycle under low-light conditions. Subjects were placed into a clear Plexiglas cylinder (38.1 cm height, 25.4 cm diameter) and allowed to naturally explore the walls by rearing up and using their forelimbs for weight support. The rats were recorded for a maximum of 5 min in the cylinder. Videotaped behavior was scored in

slow motion by an experimenter blind to the condition. A percent limb usage score was calculated using the formula $((\text{contralateral side} + 1/2 \text{ both}) / (\text{ipsilateral side} + \text{contralateral side} + \text{both})) \times 100$. A score of 50% indicates equal use of both forelimbs, whereas scores less than 50% indicate motor deficits in the contralateral forelimb. Statistical analyses included one-way ANOVA followed by Neuman–Kuels post hoc test.

Immunohistochemistry and Stereology. At 4 weeks post-lesion, the animals were sacrificed and processed for immunohistochemistry. Following perfusion with 4% paraformaldehyde, brain dissection, post-fixation, and cryoprotection, coronal sections through ventral mesencephalon were cut on a sliding microtome at thickness of 50 μm . Series of free-floating

mesencephalic sections were processed for tyrosine hydroxylase (TH; catecholamine biosynthetic enzyme and marker for midbrain dopamine cells) immunocytochemistry, as previously described (Seroogy et al. 1989; Hemmerle et al. 2014). TH immunostained cells were counted in the midbrain (substantia nigra pars compacta; SNpc) using Stereo Investigator 5.05 (MicroBrightfield, Williston, VT) utilizing unbiased stereological techniques, as detailed previously (West, 1993; Hemmerle et al. 2014; Hu et al. 2017). Contours were defined at $2.5\times$ magnification, and cells were counted at $60\times$ using the optical fractionator. The random sample sites had a grid size of 170×100 , with a guard zone of 2 μm . The coefficient of error (CE), as determined using the Gunderson correction, was ≤ 0.10 . The extent of cell loss in the SNpc was determined by comparing the

Fig. 1 Rotenone produces limited neuronal cell death, SKIP provides protection. SH-SY5Y cells were plated onto 96-well plates at a concentration of 3000 cells/well and then differentiated to neuronal-like cells with retinoic acid (10 μM) for 7 days. On the experimental day, cells were treated with 1, 10, and 100 μM of rotenone for 24 h (A) or 48 h (B). C Treatment with 10 μM of rotenone for 48 h in the presence of increasing SKIP concentrations. Mitochondrial activ-

ity measurements (cell viability) were obtained by the XTT colorimetric assay. Statistical analysis was performed by one-way ANOVA, Tukey (A and B) or LSD (C) HSD. Statistical significance is represented within the concentration group (A and B) or in relation to the rotenone group: “Rotenone 10 μM ” (C). $n = 20/\text{group}$, $*p < 0.05$, $***p < 0.001$

lesioned side to the contralateral unlesioned side. Statistical analyses included one-way ANOVA followed by Neuman–Kuels post hoc test.

Results

Rotenone Exhibits Minimal Cellular Toxicity in the Cell Viability Assay (XTT); SKIP Provides Limited Protection

Compared with control (DMSO), rotenone treatment reduced neuronal-like SH-SY5Y cell viability between 15 and 40%, depending on the dose (1, 10, 100 μ M) and duration of incubation (24 and 48 h). This is in line with the “sick but not dead” concept, thereby allowing testing for effects of SKIP (Fig. 1A, B). Surprisingly, the 100 μ M, 48-h rotenone treatment was nontoxic, although apparent consistent toxicity was observed with the lower tested concentrations. To evaluate the protective effect of SKIP against rotenone cytotoxicity, differentiated SH-SY5Y cells were exposed to 10 μ M of rotenone (exhibiting

reproducible toxic effects; Fig. 1A, B) with or without SKIP at different concentrations (10^{-15} – 10^{-6} M) for 48 h. A minor but statistically significant protective effect against rotenone toxicity was observed after treatment with 10^{-15} M SKIP (Fig. 1C).

SKIP Blunts Rotenone-Induced Decreases in Neuritic Branch Length and MT Polymerization

Rotenone was tested for reduction of neuritic length in SH-SY5Y cells at two concentrations (1 and 10 μ M) and SKIP was added (10^{-15} – 10^{-9} M), for 2 or 24 h. Whereas a 2-h exposure was either not significant or less effective (data not shown), 24-h rotenone treatment repeatedly reduced branch length by ~50% (Fig. 2A). While no SKIP protection was observed with SKIP at 10^{-15} M, 10^{-12} M, or 10^{-9} M against 10 μ M rotenone (24-h exposure period, data not shown), treatment with SKIP at 10^{-15} M or 10^{-9} M provided protection against 1 μ M rotenone (24-h exposure period; Fig. 2A). The tested 10^{-12} M SKIP did not differ from the other tested concentrations.

Fig. 2 Rotenone reduces neurite length and polymerized MT content. **A** Neurite length assessment is described in the “Methods” and the “Results”. Confocal microscopy pictures and quantitative analysis are depicted side by side. Statistical analysis was performed by one-way ANOVA, Tukey HSD. Experimental groups tested included: “Rotenone 1 μ M 2 h” group: Cont (DMSO) $n=78$; Rotenone 1 μ M $n=38$; Rotenone 1 μ M+SKIP 10^{-15} M $n=71$; Rotenone 1 μ M+SKIP 10^{-12} M $n=56$; Rotenone 1 μ M+SKIP 10^{-9} M $n=74$. “Rotenone 1 μ M 24 h” group: Cont (DMSO) $n=135$; Rotenone 1 μ M $n=122$; Rotenone 1 μ M+SKIP 10^{-15} M $n=49$; Rotenone 1 μ M+SKIP

10^{-12} M $n=54$; Rotenone 1 μ M+SKIP 10^{-9} M $n=98$. “Rotenone 10 μ M 2 h” group: Cont (DMSO) $n=215$; Rotenone 10 μ M $n=190$; Rotenone 10 μ M+SKIP 10^{-15} M $n=178$; Rotenone 10 μ M+SKIP 10^{-12} M $n=162$; Rotenone 10 μ M+SKIP 10^{-9} M $n=185$. “Rotenone 10 μ M 24 h” group: Cont (DMSO) $n=176$; Rotenone 10 μ M $n=133$; Rotenone 10 μ M+SKIP 10^{-15} M $n=110$; Rotenone 10 μ M+SKIP 10^{-12} M $n=111$; Rotenone 10 μ M+SKIP 10^{-9} M $n=114$. **B** Polymerized vs. soluble tubulin/MT assay is described in the “Methods” and “Results” section. Data were statistically analyzed by one-way ANOVA, LSD HSD ($n=2$ /group). * $p<0.05$

SKIP Protects Against Rotenone-Induced Decreases in MT Polymerization

After assessment of cell morphology (Fig. 2A), cells were harvested, and lysates were separated into polymerized (P) and soluble (S) protein fractions. Equal aliquots of each pair (P and S) were resolved on adjacent lanes by SDS polyacrylamide gel electrophoresis. The blots were probed with tubulin antibodies. The intensity of each band was quantified by Fiji and ratios were calculated by dividing the intensity value of the pelleted band over the sum of intensity values of polymerized and soluble bands [P/(S + P)]. Results after 24-h incubation with 10 μ M rotenone indicated an $\sim 30\%$ significant decrease in the relative content of polymerized MT, which was completely reversed by SKIP treatment, at all tested concentrations (10^{-15} – 10^{-9} M; Fig. 2B). Notably, in each series of experiments, we used different concentrations of rotenone to test the optimal concentration at which the specific experiment would work in the best manner. Only statistically significant results are presented. The potent rotenone and

SKIP concentrations vary between experiments, probably, because of different vulnerability of the tested cell systems to the tested drugs.

Rotenone Decreases MT Dynamics; SKIP Protects MT Speed

We aimed to evaluate the effect of rotenone on MT dynamics and of the possible protective activity of SKIP against rotenone-induced MT disruption. Differentiated neuronal-like neuroblastoma cells (N1E-115 cells) were subjected to transient transfection with an expression plasmid encoding EB3 protein, tagged to RFP (EB3-RFP). Indeed, live cell imaging of MT dynamics requires cell transfection with EB3-RFP expressing plasmid (Ivashko-Pachima et al. 2017). Here, we chose to use the easily transfected mouse neuroblastoma N1E-115 cells (Ivashko-Pachima et al. 2017, 2019b, 2021a; Ivashko-Pachima and Gozes 2019a, 2021b; Grigg et al. 2020).

Single-cell time-lapse imaging allowed us to evaluate the effect of rotenone on MT dynamics by tracking EB3-RFP

Rotenone 0.1 μ M

Fig. 3 Rotenone reduces MT dynamics, SKIP protects. Live cell imaging evaluations were carried out as described in the “Methods” and the “Results”, live imaging was conducted for 2 h, and results with 0.1 μ M rotenone are shown: upper panel—graphic calculations, lower panel—confocal imaging and track tracing (Ivashko-Pachima et al. 2017). Statistical analysis was performed by one-way ANOVA, Tukey HSD. Group used included: “Rotenone 10 μ M” group: Cont (DMSO) $n=27$; Rotenone 10 μ M $n=10$; Rotenone 10 μ M+SKIP

10^{-15} M $n=12$; Rotenone 10 μ M+SKIP 10^{-12} M $n=13$; Rotenone 10 μ M+SKIP 10^{-9} M $n=10$. “Rotenone 1 μ M” group: Cont (DMSO) $n=29$; Rotenone 1 μ M $n=14$; Rotenone 1 μ M+SKIP 10^{-15} M $n=11$; Rotenone 1 μ M+SKIP 10^{-12} M $n=11$; Rotenone 1 μ M+SKIP 10^{-9} M $n=12$. “Rotenone 0.1 μ M” group: Cont (DMSO) $n=17$; Rotenone 0.1 μ M $n=17$; Rotenone 0.1 μ M+SKIP 10^{-15} M $n=14$; Rotenone 0.1 μ M+SKIP 10^{-12} M $n=15$; Rotenone 0.1 μ M+SKIP 10^{-9} M $n=14$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Fig. 4 SKIP partially ameliorates 6-OHDA lesion-induced deficits. **A** Behavioral asymmetry scores in the cylinder test. At baseline (2 weeks after supranigral SKIP or vehicle administration, but before striatal 6-OHDA injection), all 3 groups exhibited equal usage of each forelimb (left panel). However, at 2 weeks following 6-OHDA lesions, the group pretreated with 500 pg SKIP maintained increased

use of the impaired forelimb compared to the groups pretreated with vehicle or 5 pg SKIP (right panel). **B** Stereological cell counts in the SNpc show that TH cell loss was significantly attenuated in the rats pretreated with 500 pg SKIP compared to those pretreated with 5 pg SKIP or vehicle. TH, tyrosine hydroxylase; SNpc, substantia nigra pars compacta; SX, SKIP. $n = 3\text{--}4/\text{group}$. $*p < 0.05$

comet-like structures decorating newly polymerized MT plus-ends. Time-lapse imaging followed by the 1-h treatments with rotenone alone or together with SKIP showed that rotenone in every tested concentration (0.1, 1, 10 μM) significantly reduced the track length and velocity of the EB3 comet-like structures, reflecting the lengths of the MT-growing events and the speed of MT assembly, respectively (Fig. 3 shows 0.1 μM rotenone as an example). Thus, rotenone had an impact on MT dynamics at various concentrations. However, SKIP showed protective activity only against rotenone adverse effects on the velocity of the EB3 comets, but not on the EB3 track length, most consistently at 0.1 μM rotenone concentrations (Fig. 3).

SKIP Pretreatment Partially Ameliorates 6-OHDA Lesion-Induced Deficits: Increased Behavioral Scores and Cell Survival

As indicated in the “Introduction” section, our original results indicated that NAP protects against 6-OHDA toxicity in cell cultures (Offen et al. 2000), an accepted in vitro model for PD. Given the NAP/SKIP shared mechanism of action (Amram et al. 2016; Ivashko-Pachima and Gozes 2019a), and the above rotenone results, we undertook a pilot study of SKIP efficacy in the established partial lesion 6-OHDA rat model of nigrostriatal DAergic neurodegeneration. Our results demonstrated significant effects of SKIP pretreatment in the cylinder test. At 2 weeks following 6-OHDA lesions, vehicle-treated rats exhibited substantial disuse of the impaired forelimb. Pretreatment with a one-time dose of 500 pg SKIP (but not the lower 5 pg dose) significantly

improved impaired limb usage (Fig. 4A). Similarly, stereological cell counts of surviving nigral TH-positive neurons at 4 weeks post-6-OHDA lesion revealed considerable cell loss in the vehicle-treated rats. In contrast, pretreatment of lesioned rats with 500 pg (but not 5 pg) SKIP significantly and substantially increased TH+ cell survival in the SNpc (Fig. 4B).

Discussion

The association of the pesticide rotenone and increased toxic dopaminergic metabolites has been shown before (Goldstein et al. 2015). Here, through the prism of neuroprotection, we focused on the MT-associated SKIP/rotenone and DAergic system neurotoxicity. We showed that 10^{-15} M SKIP protected against 48-h induced 10 μM rotenone toxicity, measured at the level of cellular viability (mitochondrial activity). Interestingly, high concentrations of rotenone (100 μM) were less toxic to SH-SY5Y cells, exhibiting a bell-shape toxicity effect, in agreement with the SKIP protection curve. Such a neuroprotective bell-shaped dose-response curve with a peak at 10^{-15} M was also seen before for NAP (Bassan et al. 1999). Neurite outgrowth also showed a peak at 10^{-15} M SKIP concentrations, although higher concentrations were effective as well, consonant with previous studies on NAP (Smith-Swintosky et al. 2005). MT comet speed required slightly higher concentrations, suggesting differential target engagements/downstream mechanisms. In this respect, SKIP has been shown previously to enhance ADNP-dependent axonal transport (Amram et al. 2016) and to accelerate tau-MT interaction, through EB1/3

(Ivashko-Pachima and Gozes, 2019a). These data are in agreement with NAP protecting MT-dependent axonal transport (Jouroukhin et al. 2013) and accelerating tau-MT interaction (Ivashko-Pachima et al. 2017). The converging protection of SKIP against MT disruption and DAergic-related toxicity was observed here in vivo, in an animal model of 6-OHDA-induced neurotoxicity. Interestingly, protection of the DAergic system in association with protection against tauopathy (Gozes et al. 2009) was previously suggested for NAP in another motor disease, amyotrophic lateral sclerosis (Jouroukhin et al. 2013). Moreover, as pointed out in the “Introduction” section, the ADNP system is dysregulated in PD (Chu et al. 2016).

This is the first in vivo description of SKIP protection outside of the ADNP syndrome (Amram et al. 2016), suggesting a broader range of activity/efficacy. The acute nature of the injury allows acute treatment, but with extensive pharmacodynamic properties (i.e., seeing a protective effect two weeks or even more than that, after administration). This is in accord with our NAP studies in animal models: e.g., head trauma (Beni-Adani et al. 2001; Romano et al. 2002), including pretreatment with NAP (Zaltzman et al. 2003)) and stroke (Leker et al. 2002), as well as the PD models mentioned in the “Introduction” and also in human clinical trials. Thus, in clinical trials in amnesic mild cognitive impairment (a precursor to Alzheimer’s disease), NAP administration showed efficacy even 4 weeks after cessation of treatment, suggesting an extensive pharmacodynamics effect (Gozes et al. 2009).

The present results using a pretreatment paradigm are encouraging as they demonstrate the neuroprotective potential of SKIP in a well-established model of PD. However, future experiments should examine posttreatment paradigms to be more clinically relevant, alternate routes of SKIP administration (i.e., intranasal; Fleming et al. 2011; Magen et al. 2014; Bermudez et al. 2019), higher doses of SKIP, and additional PD animal models. Future studies should also look at modified SKIP (Ivashko-Pachima and Gozes 2019a) as well as drug bioavailability and additional PD/MT-related models, toward clinical development. Lastly, we have previously shown SKIP + NAP interactions. Thus, SKIP enhanced NAP-target (EB3) interaction (affinity column chromatography) and the two peptides showed direct interaction (nuclear magnetic resonance spectroscopy) (Amram et al. 2016). These previous results are suggestive of additive or synergistic effects for SKIP + NAP, which should be further explored.

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Declarations

Conflict of Interest NAP (CP201) use is under patent protection (US patent nos. US7960334, US8618043, US10912819) and provisional patent applications; SKIP derivatives are under a composition of matter US patent, US10822375, Ramot at Tel Aviv University (IG).

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