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A novel histone deacetylase 1 and 2 isoform-specific inhibitor alleviates experimental Parkinson's disease

Author names:

Chi-Jing Choong ^a, Tsutomu Sasaki ^a, Hideki Hayakawa ^a, Toru Yasuda ^b, Kousuke Baba ^a, Yoshiyuki Hirata ^c, Shinichi Uesato ^c, and Hideki Mochizuki ^a

Affiliation:

^aDepartment of Neurology, Graduate School of Medicine, Osaka University, Yamadaoka 2-2, Suita, Osaka 565-0871, Japan

^bDepartment of Human Genetics, National Center for Child Health and Development, Okura 2-10-1, Setagaya-ku, Tokyo 157-8535, Japan

^cDepartment of Life Science and Biotechnology, Faculty of Chemistry, Materials and Bioengineering, Kansai University, Suita, Osaka 564-8680, Japan

Corresponding author: Hideki Mochizuki

Tel: +81-06-6879 3571; Fax: +81-06-6879 3571;

E-mail address: hmochizuki@neurol.med.osaka-u.ac.jp; Postal address: Department of Neurology, Yamadaoka 2-2, Suita, Osaka 565-0871, Japan.

Abstract

With increased histone deacetylase (HDAC) activity and histone hypoacetylation being implicated in neurodegeneration, HDAC inhibitors have been reported to have considerable therapeutic potential. Yet existing inhibitors lack specificity and may show substantial adverse effect. In this study, we identified a novel HDAC1/2 isoform-specific inhibitor, K560, with protective effects against MPP⁺/MPTP-induced neuronal death in both *in vitro* and *in vivo* Parkinson's disease (PD) model. K560 attenuated cell death induced by MPP⁺ in differentiated SH-SY5Y cells through the sustained expression of an anti-apoptotic protein, X-linked inhibitor of apoptosis (XIAP). Inhibition of XIAP expression by locked nucleic acid antisense oligonucleotides abolished the protective effect of K560. Inactivation of MAPK cascades, reduced p53 phosphorylation and down-regulation of p53 upregulated modulator of apoptosis (PUMA) upon K560 treatment were also observed. Furthermore, pre- and post-oral administration of K560 in mice prevented MPTP-induced loss of dopaminergic neurons in substantia nigra, suggesting that selective inhibition of HDAC1 and 2 by K560 may pave the way to new strategies for PD treatment.

Keywords

Histone deacetylase; Histone deacetylase inhibitor; Isoform-specific; K560; Parkinson's disease; Neurodegeneration; MPTP

1. Introduction

Histone acetylation, one of several epigenetic mechanisms that control gene expression, is a highly dynamic process regulated by two classes of enzyme, histone acetyltransferases (HATs) and histone deacetylases (HDACs). HAT catalyzes the acetylation of lysine residues in histone tails leading to chromatin relaxation and transcriptional activation, whereas HDAC mediates deacetylation resulting in a more condensed chromatin state and transcriptional repression (Waterborg, 2002).

In normal neuronal cells, a tightly regulated balance exists between HAT/HDAC activities to enable finely tuned control of transcription for proper gene expression regulation and maintenance of homeostasis. There is a notion that neuronal cell death occurs and pathologies develop following derangement of HAT/HDAC as histone hypoacetylation and gene repression have been extensively implicated in experimental models of numerous neurodegenerative diseases, including Huntington's disease, Friedreich's ataxia and spinal muscular atrophy (Rouaux et al., 2003; Sadri-Vakili et al., 2007; Herman et al., 2006; Kernochan et al., 2005; Shan et al., 2014).

Despite the lack of insights into the histone modifications in Parkinson's disease (PD) brains, *in vitro* overexpression studies as well as *in vivo* α -synuclein transgenic Drosophila model and mice exposed to herbicide paraquat have revealed that α -synuclein mediates neurotoxicity in the nucleus by interacting with histones and inhibiting histone acetylation (Kontopoulos et al., 2006; Goers et al., 2003). These findings thus garner interest for the exploration of HDAC inhibitors to restore imbalance between HAT/HDACs as potential therapies for PD.

HDACs are grouped into four classes, which are further classified into 18 isoforms on the basis of their size, cellular localization, number of catalytic active sites and protein homology. Several HDAC inhibitors including suberoylanilide hydroxamic acid (SAHA), trichostatin A (TSA), valproic acid (VPA) and sodium butyrate are shown to be efficacious in cell culture and animal models induced by mitochondrial toxins, including 1-methyl-4-phenylpyridinium (MPP⁺), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), rotenone and paraquat (Kidd and Schneider, 2010; Kidd and Scheider, 2011; Chen et al., 2012; Monti et al., 2010). However, these pan inhibitors of HDAC classes I and II lack specificity and may cause undesired side effects by inhibition of non-essentially inhibited HDAC classes. This is evidenced by the fact that despite its extensive clinical use and well-tolerated pharmacokinetic properties, valproic acid did not significantly alter any Parkinsonian feature in clinical setting but augmented dyskinesia (Nutt et al., 1979). Attempt to elude deleterious side effect while maintaining the neuroprotective ability of HDAC inhibitor would require improved selectivity for specific HDACs.

In this present study, we screened our compound library in search for novel isoform-specific HDAC inhibitor that shows neuroprotective effect in experimental PD.

2. Materials and methods

2.1 Cell culture and differentiation

Human neuroblastoma cell line SH-SY5Y (ATCC CRL-2266) was cultured in DMEM (Sigma-Aldrich) supplemented with 10% FBS (Gibco) at 37 °C in a 95% air, 5% CO₂ humidified incubator. Cells were routinely subcultured when confluent. For differentiation, cells were cultured in DMEM containing 3% FBS and 10 µM retinoic acid (Sigma-Aldrich) for eight days. The differentiation medium was changed every three to four days.

2.2 MPP⁺ and HDAC inhibitor treatment paradigm

MPP⁺ (Sigma-Aldrich) was dissolved at the stock concentration of 200 mM in sterilized water. Freshly prepared MPP⁺ or vehicle, water were added to the differentiated SH-SY5Y cells and incubated for 48 hr. Apicidin, TSA, SAHA and VPA, were purchased from Sigma-Aldrich. Compound K32, K120, K164, K310, K330, K430, K560 (>99% purity by HPLC analysis), compound 11, also known as Octanedioic acid (2-aminophenyl)amide-[3-(1-phenyl-1H-[1,2,3]triazol-4-yl)phenyl]amide and MS-275 were prepared in the laboratory of Prof. S. Uesato (Kansai University, Japan). These HDAC inhibitors are grouped according to their chemical class and HDAC selectivity, as listed in Supplementary Table 1. Co-treatment of these HDAC inhibitors was tested for protective effect with dimethyl sulfoxide (DMSO) (Sigma-Aldrich) as mock control.

2.3 Lactate dehydrogenase-based cytotoxicity assay

Cell death after 48 hr exposure to MPP⁺ was assessed by assaying lactate dehydrogenase (LDH) activity using a cytotoxicity detection kit (Roche). Collected culture medium was centrifuged at 300 x g for 5 min before assaying according to the manufacturer's instruction. Released LDH reduced the tetrazolium salt to a red-colored formazan salt. The amount of formazan, which correlated directly with the amount of LDH activity, was quantified by absorbance at a wavelength of 490 nm.

2.4 Immunostaining for active caspase 3

Cells were fixed with 4% paraformaldehyde for 30 min at room temperature. After permeabilization with 0.5% Triton X-100 solution for 10 min and washing in phosphate-buffered saline (PBS), cells were incubated in PBS with 10% blockace (Yukizirushi) for 1 hr and subsequently with primary anti-active caspase 3 (BD Pharmingen TM) for 1 hr with

shaking at room temperature. After several washes in PBS, the cells were incubated with CyTM3-conjugated anti-rabbit antibody (Jackson ImmunoResearch) for 1 hr at room temperature. After washing in PBS, the cells were counterstained with DAPI mounting medium (Vectashield) and were observed microscopically using BZ-9000 (Bioevo, Keyence) all-in-one fluorescence microscope. Percentage of active caspase 3 positive cells of total cells was quantified by counting cells from ten random low magnification fields.

2.5 Determination of HDAC activity

HDAC activity assay was performed as per the manufacturer's protocol provided with the FLUOR DE LYS[®]-Green HDAC fluorometric activity assay kit (Enzo Life Sciences).

2.6 HDAC2 promoter assay

Differentiated SH-SY5Y cells were plated in 24-well plates and were transduced with adenoviruses generated from pGL4 luciferase reporter vector containing HDAC2 promoter (-1500bp) with adenoviruses generated from an empty reporter vector as control. Adeno-TK-Luc Renilla luciferase was used as internal control (Promega). Medium change was performed three hours after virus transduction and cells were incubated for 48 hr before 2 mM MPP⁺ was added. Cell samples were collected in passive lysis buffer at specified time points. Luciferase activity was measured with a luminometer using the Dual-Luciferase reporter assay system (Promega) according to the manufacturer's instructions.

2.7 HDAC-GloTM 2 assay

HDAC-GloTM 2 assay (Promega) was used to measure the relative activity of HDAC2 enzyme from cells. SH-SY5Y cells were seeded into a white 96-well plate and were subjected to differentiation. Differentiated cells were treated with twofold serial dilutions of

SAHA control, K560 and MPP⁺ for 48 hr before assaying according to the manufacturer's instruction.

2.8 Human apoptosis antibody array

Proteome Profiler™ Human apoptosis antibody array (R&D Systems) was performed according to the manufacture's manual for detection of changes of relative expression levels of 35 apoptosis-related proteins in control and treated cells. Briefly, cell lysates (200 µg total protein each) were applied to be incubated overnight with antibody array membrane. The membranes were then washed and cocktail of biotinylated antibody mix was used to detect apoptosis-related proteins. After incubation with HRP-conjugated streptavidin, signals were visualized by chemiluminescence. Average signal (pixel density) of the pair of duplicate spots representing each apoptosis-related protein was determined with average background signal being subtracted from each point. Comparison of corresponding signals in different array membranes reveal relative change in apoptosis relative protein levels between samples.

2.9 Western blotting analysis

Cells were harvested and lysed in CellLytic™ MT Cell Lysis Reagent (Sigma-Aldrich) with a protease inhibitor (Calbiochem) and phosphatase inhibitor (Merck Millipore) mixture set. Protein concentration of cell lysates was determined using a BCA protein assay kit (Pierce). Lysates at the amount of 10 µg/lane were resolved on 5-20% gradient polyacrylamide gel (Atto) and transferred to PVDF membranes (Millipore). The membranes were first blocked in EzBlock Chemi (Atto) and then probed with primary antibodies: acetyl histone H3 Lys9 (MBL), HDAC1, HDAC2 (Abcam), p53, PUMA, SAPK/JNK, phospho-SAPK/JNK (T183/Y185), XIAP, phospho-MEK1/2, ERK1/2 (Cell Signalling), phospho-Raf (ser259) (New England Biolabs), actin (Millipore), followed by secondary HRP-conjugated anti-

mouse IgG or anti-rabbit IgG (GE Healthcare). Protein bands were detected using ECL Prime Western Blotting Detection System (Amersham).

2.10 XIAP silencing by locked nucleic acid oligonucleotides transfection

Two chemically synthesized locked nucleic acid oligonucleotide sequences targeting XIAP (XIAP 1 LNA longRNATM GapmeR: 5'-CCGTTGATAAATCTG-3', XIAP 2 LNA longRNATM GapmeR: 5'-GGTTAGGTGAGCATA-3') and a 5'-fluorescein-labeled oligonucleotide sequence (5'-56-FAM/AACACGTCTATACGC-3') which serves as negative control were purchased from Exiqon (Vedbaek, Denmark). Cells were transfected at 70% confluence with oligonucleotides diluted in sterile water at final concentration of 200 nM using Lipofectamine RNAi MAX (Invitrogen) according to the manufacturer's instructions. Twenty-four hour post transfection, cells were subjected to MPP⁺ and K560 treatments. LDH activity was assayed for cell death evaluation 48 hr later.

2.11 Inhibition of MAP Kinases

SP600125 (Sigma-Aldrich), a JNK inhibitor and U0126 (Sigma-Aldrich), a MEK inhibitor were added to the differentiated SH-SY5Y at 1 μ M and 5 μ M respectively in the absence or presence of MPP⁺. DMSO was used as a vehicle control for both inhibitors.

2.12 Animals

Studies were conducted in male C57BL/6 mice (8-10 weeks old; weighing 20-25 g, Charles River Laboratories). Animals were kept four per cage under 12 hr light/dark cycle and standard housing condition with ad libitum access to food and water for 1 week before the experiment. All animal experiments have been conducted according to the Osaka University Medical School Guideline for the Care and Use of Laboratory Animals. Animal surgeries and

experimental procedure were approved by the Osaka University Medical School Animal Care and Use Committee.

2.13 Liquid chromatography-electrospray ionization tandem mass/mass spectrometry (HPLC-ESI-MS/MS) for the quantification of K560 in the brain

Distribution of K560 into the brain was measured using quadrupole mass spectrometry. Mice were treated with oral administration of K560 dissolved in ultrapure laboratory grade water containing 0.1% Tween 80 (TCI) at 45 and 80 mg/kg/day for a week. Harvested brains were added to MeCN/H₂O (1:1100 μ l), and then homogenized with pestle (Nippi co., ltd), followed by centrifugation at 12,000 rpm at 4 °C for 15 min. Collected supernatant was filtrated to remove discarded tissue before being analyzed using HPLC-ESI-LC-MS/MS (Prominence system, Shimadzu co., ltd and Q Exactive series, Thermo scientific co., ltd). High-performance liquid chromatography (HPLC) method was based on the use of a reversed-phase C8 column (XTerra® MS C8 column, 2.1×150 mm, Waters co., ltd) and the solvent, MeCN:H₂O with 0.1% formic acid (Wako Pure Chemical co., ltd). Flow rate was 0.2 mL/min and column temperature was set at 40 °C. Multiple Reaction Monitoring (MRM) quantitation was performed.

2.14 MPTP and K560 administration

Mice were divided into four groups ($n \geq 3$ each): none/saline, K560/saline, none/MPTP, K560/MPTP. For pre-treatment, mice were subjected to oral administration of K560 dissolved in ultrapure laboratory grade water containing 0.1% Tween 80 at 45 mg/kg/day for seven consecutive days. MPTP (Fluka) injection began two days after the first oral administration of K560. MPTP was injected intraperitoneally at 30 mg/kg/day for five consecutive days and at 30 min after each oral administration of K560. In the control group,

mice were fed with water instead of K560 and were injected with equal volume of saline (Otsuka) instead of MPTP. For post-treatment, oral administration of K560 began after the day of last MPTP injection. Guidelines for handling MPTP were strictly followed as described previously (Jackson-Lewis and Przedborski, 2007).

2.15 Immunohistochemistry

Mice were anesthetized, perfused transcardially with PBS and 4% paraformaldehyde and decapitated. After brain sample collection, striatal and serial nigral coronal sections of 20 μ m thickness were prepared for immunohistochemical study as previously reported (Furuya et al., 2004).

2.16 Stereological tyrosine hydroxylase (TH)-positive cell counting

To assay changes in the number of dopaminergic neurons in the substantia nigra pars compacta (SNpc), the total number of TH- and Nissl-double-positive neurons in every fourth section through the entire extent of the SNpc was counted based on the method of Furuya et al. (2004).

2.17 Immunoblotting

The mice were perfused transcardially with PBS and ventral midbrain and striatum tissues were removed respectively. Total protein extracts of the ventral midbrain were obtained by homogenization in CellLytic-MT mammalian tissue lysis/extraction reagent (Sigma-Aldrich) with protease inhibitor and phosphatase inhibitor mixture. Determination of protein concentration in the tissue lysates and western blot analysis was carried out as mentioned above.

2.18 Statistical analysis

Data are expressed as mean $\pm$ S.E.M. Statistical analysis was performed using one-way-ANOVA (SPSS). A p value of less than 0.05 denotes a statistically significant difference.

3. Result

We identified a novel isoform-specific HDAC inhibitor with selective inhibition for HDAC1 and 2, K560 from our library that showed neuroprotective effect in experimental PD, both *in vitro* differentiated SH-SY5Y model of MPP⁺ toxicity and *in vivo* MPTP mouse model.

3.1 K560 attenuates MPP⁺-induced cytotoxicity and caspase 3 activation *in vitro*

Retinoic acid-differentiated SH-SY5Y cells have been shown to acquire dopaminergic-like phenotype by transcriptional profiling (Korecka et al., 2013) and hence were deemed suitable to be used as *in vitro* PD model in this study. Differentiated SH-SY5Y cells were treated with varying concentration of MPP⁺ 0.5, 1, 2 mM for 48 hr. The effect of co-treatment with several novel isoform specific HDAC inhibitors of hydroxamate, carboxylate, 2-aminobenzamide and thienyl substituted 2-aminobenzamide types were assessed with TSA, SAHA, VPA and MS-275 as reference compounds. LDH assay for cell viability assessment revealed that MPP⁺ markedly induced SH-SY5Y cell death in a concentration-dependent manner. Surprisingly, TSA and SAHA did not prevent while VPA exacerbated MPP⁺-induced cell death. Among the tested compounds from our HDAC inhibitor library, 1 μ M K560 treated cells (14.73 ± 0.52) showed significantly greater resistance to 2 mM MPP⁺-induced cell death compared to mock cells (22.62 ± 0.53) ($p=0.008$), comparable to that of MS-275 (Figure 1a). K560, a HDAC1/2 isoform specific inhibitor, was selected and subjected to further validation and exploitation. Cell death assessment data was further supported by active caspase 3 immunostaining. Figure 1b displays representative images of

caspase 3-immunoreactive cells with their nuclei counterstained by the blue fluorescent nuclear stain DAPI. K560 treated cells showed significantly less percentage of active caspase 3 positive cells of total cells (8.88 ± 1.41 ; 16.67 ± 1.54) than control (13.36 ± 1.99 ; 27.24 ± 3.59) upon 1 and 2 mM MPP⁺ treatment ($p=0.043$) (Figure 1c). These data demonstrate K560 protects against MPP⁺-induced cytotoxicity and caspase 3 activation in differentiated SH-SY5Y cells.

3.2 Changes of HDAC1 and 2 expression and activity upon K560 and MPP⁺ treatment

K560, a 2-amino-5-(thiophen-2-yl)benzamide-series HDAC inhibitor, has a 4-ethyl-2,3-dioxopiperazine-1-carboxamide group as a surface recognition domain and a 2-aminobenzamide moiety that exerts HDAC1/2-selective inhibition by projecting the 2-thienyl ring into extensive internal cavity adjacent to the zinc binding site within the HDAC1/2 pocket (Figure 2a) (Hirata et al., 2012). We demonstrated selective HDAC1/2 inhibition of K560 in SH-SY5Y cells using FLUOR DE LYS[®]-Green HDAC fluorometric activity assay. K560 inhibited the activities of HDAC1 (IC₅₀: 0.09 μ M) and HDAC2 (IC₅₀: 0.52 μ M), but not that of HDAC3 (IC₅₀: >100 μ M) in SH-SY5Y cells. We also confirmed by western blotting that administration of K560 induced significant hyperacetylation of histone H3 at lysine 9 in SH-SY5Y cells ($p=0.001$) (Figure 2b-c). We then examined whether levels of HDAC1 and 2 protein expressions are altered in SH-SY5Y cells by K560 and MPP⁺ treatment. HDAC1 protein expression merely showed a tendency of being upregulated upon MPP⁺ treatment but was significantly suppressed by K560 addition. Interestingly, HDAC2 expression remained unchanged upon 1 μ M K560 addition but was significantly increased by 2 mM MPP⁺ compared to controls (Figure 2d-e). This was further confirmed by adenovirus-mediated HDAC2 promoter assay in which HDAC2 promoter activation was observed in SH-SY5Y cells as soon as 12 hour after MPP⁺ was added, implying enhanced transcriptional

activity of HDAC2 (Figure 2f). We next tested the effect of K560 and MPP⁺ on HDAC2 deacetylation activity in SH-SY5Y cells using HDAC-Glo2 assay. In a concentration-dependent manner, SAHA (a class I and II HDAC inhibitor which serves as control) and K560 exhibited potent inhibitory activity against HDAC2 enzyme while MPP⁺ treatment led to increased HDAC2 enzyme activity (Figure 2g). These data indicate K560 with potent HDAC2 inhibitory property may reverse the imbalance caused by MPP⁺ treatment.

To rule out the need of HDAC3 inhibition, we evaluated the effect of an HDAC3 selective inhibitor, compound 11, and an HDAC2 and 3 selective inhibitor, Apicidin (Chen et al., 2009; Khan et al., 2008). Compound 11 exerted no significant protective effect while Apicidin induced strong neurotoxic effect at 100 nM and 1 μ M (Supplementary Figure 1a-b), suggesting that selective inhibition of HDAC1 and 2 may be adequate to protect against MPP⁺-induced toxicity.

3.3 Sustained high-level expression of XIAP by K560 reduced MPP⁺-induced apoptosis

Cell death assessment data demonstrated that K560 reduced MPP⁺-induced apoptosis. A human apoptosis antibody array was used to further elucidate the mechanisms underlying the protective role of K560 against MPP⁺-induced apoptosis in differentiated SH-SY5Y cells (Figure 3a). Differentiated SH-SY5Y cells were subjected to 2 mM MPP⁺ stimuli in the presence or absence of 1 μ M K560. Cell extracts were prepared and analyzed. Human apoptosis antibody array showed that 5 out of a total of 35 apoptosis-related protein screened showed significant changes following MPP⁺ stimuli and K560 treatment either reversed or prevented such changes. MPP⁺ addition caused reduction of XIAP, livin, caspase and increased p53 phosphorylation at S15 and S46. K560 treated cells were found to maintain

high level of XIAP (0.90 ± 0.06) and livin (0.22 ± 0.008) as compared to control (0.50 ± 0.04 ; 0.12 ± 0.004) after MPP⁺ addition ($p=0.017$; $p=0.003$). Higher level of caspase-3 (0.42 ± 0.006) was also detected in K560 treated cells than control upon MPP⁺ stimuli (0.32 ± 0.004 ; $p=0.002$). Diminished p53 phosphorylation can be observed at S15 ($p=0.011$) and S46 ($p=0.021$) with K560 treatment (Figure 3b). These data suggest K560 exerts protection against MPP⁺-induced cell death through the manipulation of these few key apoptosis-related molecules.

3.4 Suppression of XIAP abolishes the protective effect of K560

Consistent with the data of apoptosis antibody array, western blot analysis demonstrated sustained high level of XIAP in K560 treated cells even after MPP⁺ treatment ($p=0.038$) (Figure 4a-b). To elucidate if the protective effect conferred by K560 is dependent on XIAP, we transfected SH-SY5Y cells with XIAP-targeting LNA antisense oligonucleotides (XIAP 1 and XIAP 2 LNA longRNATM GapmeR) at 200 nM for XIAP silencing before subjecting them to K560 and MPP⁺ treatment. Efficiency of LNA oligonucleotide transfection was confirmed by detection of green fluorescence in approximately 50% of the cells transfected with 5'-fluorescein-labeled negative control oligonucleotide. Untransfected cells showed no fluorescence (Figure 4c). Western blot analysis verified down-regulation of XIAP protein expression in SH-SY5Y cells following transfection with XIAP-targeting LNA antisense oligonucleotides (Figure 4d). Inhibition of XIAP expression by both LNA sequences sensitized the cells to MPP⁺ toxicity (28.85 ± 2.49 , $p=0.002$; 39.13 ± 0.74 , $p=0.0004$), with the addition of XIAP 2 LNA longRNATM GapmeR posing greater damage to cells than control (22.16 ± 1.61). More importantly, LNA antisense oligonucleotides-mediated silencing of XIAP also abolished the protective effect of K560 (Figure 4e). Collectively, these data

indicate that K560 mediates protective effect against MPP⁺ toxicity in differentiated SH-SY5Y cells in a XIAP-dependent manner.

3.5 K560 blocks activation Raf-MEK-ERK cascades

Aside from the major regulator XIAP, we postulated there are other molecules or pathways that act in a parallel and/or convergent manner to effect changes in cellular response upon MPP⁺ and K560 addition. With the apoptosis antibody array showing reduced p53 phosphorylation by K560 in response to MPP⁺ stimuli (Figure 3), we went on to examine the protein levels of total p53 and a p53 downstream target, p53 upregulated modulator of apoptosis (PUMA). MPP⁺ treatment led to elevated p53 expression but the protein level was not altered upon K560 addition. In contrast, MPP⁺-induced up-regulation of PUMA was impeded by K560 treatment ($p=0.015$) (Figure 5a(i-iii)). These results reveal that in differentiated SH-SY5Y cells, MPP⁺ triggers p53-dependent apoptotic cell death and K560 suppresses p53 activation by hindering its phosphorylation without affecting its protein level and subsequently leads to a decline of p53-inducible proapoptotic gene PUMA.

We also evaluated the effect of K560 on the changes of the diverse stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) and mitogen-activated protein kinase (MAPK) pathways. K560 treated cells showed reduced phosphorylation of SAPK/JNK both at basal level and after MPP⁺ addition compared to control cells ($p=0.019$) (Figure 5b(i-ii)). SP600125, a JNK inhibitor, at 1 μ M, did not adequately suppress MPP⁺-induced cell death (Figure 5b(iii)). These findings imply that activation of SAPK/JNK may not be the direct cause for MPP⁺ toxicity and K560-mediated SAPK/JNK inactivation may not largely contribute to its protective effect against MPP⁺ toxicity.

The MAPK pathway includes the signaling molecules Raf, MEK, and ERK. MPP⁺-induced activation of Raf, MEK and ERK1/2, as evidenced by increased phosphorylation, was significantly counteracted by K560 ($p=0.015$, $p=0.025$, $p=0.006$) (Figure 5c(i-iv)). Inhibition of MAPK pathway using a potent MEK-specific inhibitor, U0126 at 5 μ M, mitigated MPP⁺ toxicity, confirming Raf-MEK-ERK signaling pathway activation as key modulator in cell death mediated by MPP⁺ ($p=0.07$) (Figure 5c(v)). These data demonstrate that inactivation of Raf-MEK-ERK cascades at least partly mediates the protective effect of K560 against MPP⁺ toxicity in differentiated SH-SY5Y cells.

3.6 Effects of K560 on MPTP-induced loss of dopaminergic neurons in mice

We then proceeded to assess whether K560 can protect dopaminergic neurons in subacute MPTP-lesioned mouse model which recapitulates many of the pathogenetic processes operative in PD. Figure 6a illustrates the experimental schedule of K560 and MPTP treatment. K560 was orally administered to C57BL/6 mice at 45 mg/kg for seven consecutive days, starting two days before the first MPTP injection. MPTP was administered intraperitoneally at 30 mg/kg/day for five consecutive days. Western blotting and immunohistochemistry analysis were conducted using brain samples collected on both day 2 and 21 after the last MPTP injection.

We obtained evidence for penetration of K560 across blood-brain barrier into the brain of the mice and also quantified its concentration in the brain through the HPLC-ESI-MS/MS analysis. Mass spectrogram of the MeCN/H₂O extract from the homogenized brain detected a parent peak corresponding to K560 at m/z : 492.1701, whose MS/MS spectrum unequivocally indicated its daughter ion at m/z : 350.0240. Concentration of K560 in the brain was determined utilizing the calibration curve of K560 concentration-parent peak intensity.

Western blot analysis showed increased level of histone H3 acetylation in the brain of mice subjected to oral administration of K560 at 45 mg/kg and 80 mg/kg (Figure 6b). When the increase in acetyl histone H3 content elicited by K560 is compared with that elicited by MS-275 (Simonini et al., 2005), K560 appears to be more potent than MS-275.

Subacute regime of MPTP damages SNpc dopaminergic neurons in a time-dependent manner with mitochondrial-dependent apoptotic neuronal death culminating between days 2 and 4 after the last MPTP injection (Vila et al., 2001). Our data showed MPTP treatment resulted in a significant reduction of TH and Nissl double-positive cells in the SNpc compared with saline-treated age-matched control mice on both day 2 and 21. K560 treated mice showed merely a tendency to attenuate MPTP-induced loss of TH- and Nissl-double-positive neurons on day 2 post MPTP, a time point towards the peak of MPTP-induced apoptotic cell death but exhibited a marked attenuation of cell loss on day 21 when the MPTP-induced lesion had been stabilized ($p=0.02$) (Figure 6c-d). Correspondingly, western blot analyses showed high constitutive expression levels of TH protein in the SNpc of saline injected mice. MPTP resulted in a dramatic down-regulation of TH protein in the same brain region. K560 treatment significantly reverted the decrease in TH level induced by MPTP on day 21. K560 treatment also reduced XIAP protein depletion upon MPTP treatment significantly on day 2 ($p=0.016$) but only showed such tendency on day 21 (Figure 6(e)). These results indicate that K560 pre-treatment attenuates the dopaminergic neurotoxicity induced by MPTP and XIAP partially contributes to its protective effect.

To determine whether K560 pre-treatment affected the integrity of striatal dopaminergic fibers, the density of TH-immunoreactivity in striata was assessed. MPTP treatment induced loss of dopaminergic nerve terminal in the striatum on both day 2 and 21 after the last

injection. K560 pre-treatment tended to protect against such decrease on day 2 but not day 21 after last MPTP injection, albeit insignificantly (Supplementary Figure 2). These data suggest that the dopaminergic nerve terminals are more affected than cell bodies in agreement with several previous reports (Liberatore et al., 1999; Wu et al., 2002).

We also evaluated the effect of post-MPTP treatment with HDAC inhibitor K560 as this would be more akin to the treatment condition in patients with PD. In the post-MPTP treatment with K560, mice received single injection of 30 mg/kg MPTP for five consecutive days and daily administration of K560 (45 mg/kg) for a week starting after the day of last MPTP injection (Supplementary Figure 3a). Post-MPTP treatment with K560 significantly mitigated MPTP-induced decrease in TH- and Nissl-double-positive cell count ($p=0.033$) and TH protein levels ($p=0.039$) respectively in the SNpc than that of control, although to a lesser extent when K560 was used as a pre-treatment (Supplementary 3b-d). Our results demonstrate that both pre- and post-MPTP treatment with K560 confer protection against dopaminergic neuron degeneration.

4. Discussion

Here we provide evidence that a novel HDAC1/2 isoform-specific inhibitor, K560, protected against MPP⁺/MPTP-mediated neuronal death with sustained high-level expression of XIAP in *in vitro* and *in vivo* Parkinson's disease (PD) model. K560 conferred dopaminergic neuroprotection not only by pre- but also by post-treatment, implying a potential application of K560-based therapy for PD.

Most HDAC inhibitors comprise a hydroxamic acid or 2-aminobenzamide moiety as a zinc ion-chelating domain. The former compounds, typified by TSA (Yoshida et al., 1990;

Yoshida et al., 1995) and SAHA (Finnin et al., 1999; Kelly et al., 2003), are broad-ranging inhibitors of HDACs, whereas the latter, typified by MS-275 (Suzuki et al., 1999), is an orally available inhibitor that targets HDAC 1, 2 and 3. MS-275 has been reported to be 100-fold more potent than VPA in increasing acetyl histone H3 expression in the frontal cortex, and its action is longer lasting than that of VPA (Simonnini et al., 2005). K560 has been shown to have a promising pharmacological profile comparable to that of MS-275 in addition to no weight loss observed in treated mice (Hirata et al., 2012).

HDAC1 exhibits high degree of homology to HDAC2 (85% of global sequence identity) and has a high degree of functional overlap with HDAC2 for many biological processes (Gregorette et al., 2004). Gene knockout studies of HDAC1, HDAC2, and HDAC3 in mice have shown that each of these enzymes has crucial developmental and physiological role. Combined deletion of HDAC1 and HDAC2 leads to severely impaired brain development and embryonic lethality while HDAC3 deletion displays severe neurological deficits and premature death (Hagelkruys et al., 2014; Norwood et al., 2014). However, one must admit genetic studies especially constitutive global germline knockouts are not the same as pharmacological studies where the dosages and length of duration of drugs can be altered. Indeed, elevating HDAC1 expression by ectopic expression promotes the death of healthy cerebellar granule neurons and cortical neurons in culture and the neurotoxic effect of HDAC1 requires interaction and cooperation with HDAC3. Knockdown of HDAC1 suppresses HDAC3 neurotoxicity and vice versa (Bardai et al., 2012). A cytotoxic role of HDAC1 upon nuclear export has also been linked to a mechanism impairing mitochondrial transport in damaged neurons (Kim et al., 2010). On the other hand, HDAC2 has been reported to negatively regulate expression of genes important for learning and memory. Reversing the build-up of HDAC2 by short-hairpin-RNA-mediated knockdown reinstates

structural and synaptic plasticity, and abolishes neurodegeneration-associated memory impairments (Guan et al., 2009; Graff et al., 2012). HDAC2 has also been reported to be involved in oxidative stress-induced neuronal apoptosis through regulation of Foxo3a-mediated gene transcription (Peng et al., 2015). Lebrun-Julien and Suter (2015) demonstrated tamoxifen-induced, conditional combined HDAC1 and HDAC2 ablation promotes survival of retinal ganglion cell after injury. Specific HDAC1/2 ablation inhibited apoptosis strongly activated in axotomized mouse retinal ganglion cell by impairing the crucial acetylation status of p53 and reducing PUMA expression. Similarly, in our present *in vitro* study, we found that HDAC1/2 inhibitor K560 treatment suppressed PUMA expression which was increased upon MPP⁺ treatment in SH-SY5Y cells. We also demonstrated that MPP⁺ increased expression and also induced activation of HDAC2 and the neuroprotection conferred by K560 may be attributed to its suppression of MPP⁺-induced increase of HDAC2 activity.

Although HDAC3 has been shown to have selective toxicity in neurons and play a role in mutant Htt-induced neurodegeneration (Bardai et al., 2011; Bardai et al., 2013), genetic knockdown of HDAC3 does not modify disease-related phenotypes in a mouse model of Huntington's disease (Moumne et al., 2012). Importantly, cautionary evidence showed that depleting HDAC3 in post-mitotic neurons can be rather deleterious (Venkatraman et al., 2014). The role of HDAC3 in suppressing p53-dependent apoptosis has also been recently emphasized (Choi et al., 2015b). This happens to be the case in Parkinson's disease model. Choi et al. (2015a) demonstrated PTEN-induced putative kinase 1 (PINK1), which is linked to autosomal recessive early-onset familial Parkinson's disease, mediates phosphorylation of HDAC3, enhances its direct association with p53 and causes subsequent hypoacetylation of p53 to suppress dopaminergic neuronal cell death. Notably, there was no significant

neuroprotective effect of HDAC3 selective inhibitor in our *in vitro* model. These notions highlight the advantage of K560 as a selective inhibitor of HDAC1 and 2.

However, Collins et al. (2015) recently reported specific class IIa MC1568 HDAC inhibitor to be effective in promoting dopaminergic neurite growth and protecting neurons and neurite arbours from the neurotoxic effects of MPP⁺ *in vitro*. Furthermore, HDAC6 appears as a key modulator of cell protective response to the cytotoxic α -synuclein aggregates through induction of aggresome-like inclusion formation and heat shock response (Su et al., 2011; Du et al., 2010; Du et al., 2014; Kawaguchi et al., 2003). These results are in favor of a neuroprotective role of HDAC6 in PD rather than a neurotoxic role as seen in other models including AD, HD and ALS (Govindarajan et al., 2013; Taes et al., 2013; Bobrowska et al., 2011). Taken together, HDAC1 and 2 may not be the only HDAC targets in PD model and the optimal HDAC targets *in vivo* therefore remain uncertain.

In this study, we failed to observe protective effect of TSA, SAHA and VPA in our *in vitro* model, consistent with previous studies reporting that VPA induces apoptosis in differentiating hippocampal neurons and TSA inhibits survival of dopaminergic neurons (Wang et al., 2009; Wang et al., 2011). A temporally defined treatment regimen involving pulse inhibition may avoid such toxic effect (Langley et al., 2008). Favorably, our data indicated K560 attenuated MPP⁺-induced neuronal death even with prolonged treatment, signifying its lack of basal cytotoxicity. Treatments with TSA, SAHA and VPA have also all been associated with an upregulation of neurotrophic factor expression such as glial cell line-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) from astrocytes as well as inhibition of pro-inflammatory cytokine release from microglia. Such neurotrophic factors mediate the HDAC inhibitor-induced neurotrophic effects of preserving

dopaminergic neuronal function as well as protecting dopaminergic neurons from lipopolysaccharide- or MPP⁺-mediated neurotoxicity in mesencephalic neuron-glia cultures (Chen et al., 2007; Chen et al., 2006; Wu et al., 2008; Chen et al., 2012). This may explain why we could not observe protective effect of TSA, SAHA and VPA in our homogeneous neuronal cultures. Moreover, two recent reports highlighted that hydroxamate-based HDAC inhibitors can mediate neuroprotection independent of HDAC inhibition. Sleiman et al. (2014) reported protective effects of hydroxamic acid-containing small molecules are likely unrelated to direct epigenetic regulation via HDAC inhibition, but rather due to their ability to bind metals. While Olson et al. (2015) found that a subset of hydroxamate-based HDAC inhibitors form active catalase mimetics capable of protecting neurons from exogenous hydrogen peroxides, underscoring the need for careful structure-activity relationship studies when using pharmacological approaches.

We observed following K560 treatment, high XIAP expression level is retained upon MPP⁺ insult in *in vitro* differentiated SH-SY5Y cell model and *in vivo* MPTP mouse model. Inhibitors of apoptosis (IAPs) are a family of highly conserved proteins that regulate cell survival through binding to caspases, the final executioners of apoptosis. XIAP is the most widely expressed IAP and plays an important function in regulating cell survival (Deveraux et al., 1997). Deregulation of antiapoptotic activity of XIAP has been implicated in neurodegenerative disorders. XIAP has been observed to be S-nitrosylated by nitrosative stress in MPTP model of PD and also in the caudate of PD patients (Tsang et al., 2009). This S-nitrosylation of XIAP directly compromises its anti-caspase 3 and anti-apoptotic functions which thereby affect the survival of neurons in the pathogenesis of PD. Multiple lines of evidence from earlier investigations has shed light on the neuroprotective role of XIAP overexpression in the MPTP model. Eberhardt et al. (2000) showed that adenoviral gene

transfer of XIAP prevented dopaminergic neuronal death induced by MPTP or its active metabolite MPP⁺ *in vitro* and *in vivo* but the protection is not associated with a preservation of nigrostriatal terminals. Crocker et al. (2003) then demonstrated that nigrostriatal dopamine neurons of novel transgenic XIAP mice were resistant to the damaging effects of MPTP. Interestingly, MPTP-induced reduction of striatal dopamine metabolism and behavioral impairment were also attenuated in these transgenic XIAP mice. Taken together, these findings suggest that strategies such as the use of HDAC inhibitor K560 to enhance neuronal expression of XIAP may provide therapeutic benefit for the treatment of neurodegeneration in PD.

We also detected activation of Raf, MEK and ERK upon MPP⁺ treatment and K560 conferred protection by counteracting these activations. Despite being well characterized to be involved in cell survival, activation of ERK1/2 has been recently shown to contribute to cell death under certain conditions implicating dual roles for ERK1/2 (Zhuang and Schnellmann, 2006). Zhu et al. (2012) reported that in chronic MPP⁺ cell model, inhibiting activation of ERK conferred complete cytoprotection with reversal of mitochondrial injury. Given our *in vitro* data, we speculate that inhibition of HDAC1 and 2 by K560 reduces the deacetylation of histones, which leads to chromatin relaxation and activation of multiple gene products acting in a parallel and/or convergent manner conducive to neuroprotection.

We proceeded to assess *in vivo* efficacy of K560 in protecting dopaminergic neurons against MPTP-induced cell death in a mouse model of PD. There are three widely used MPTP regimens: acute, subacute and chronic (Furuya et al., 2004; Yasuda et al., 2011). A previous study has reported that both acute and subacute MPTP injection induced TH-positive dopaminergic neuronal cell death in the SNpc. However, inflammation mediated by

microglial activation, up-regulation of IL-1 β and caspase 11 occurred solely in the SNpc and striatum of the acute, but not subacute MPTP model (Furuya et al., 2004). Overexpression of the Apaf-1 dominant-negative inhibitor inhibited the mitochondrial apoptotic cascade in subacute MPTP treatment but not in acute MPTP treatment (Mochizuki et al., 2001). Considering the differences in the acute and subacute MPTP mice models and also our *in vitro* data that mainly points to the anti-apoptotic properties of K560, we have opted for subacute MPTP that is seemingly a better model to examine neuroprotective effect of K560. MPTP significantly induced striatal dopaminergic denervation compared with the saline control group. Despite its ability to protect the dopaminergic cell bodies in both pre- and post-treatments, K560 failed to preserve striatal nerve fibers from MPTP neurotoxicity for the reason that dopaminergic nerve terminals are reportedly more persistently affected than the cell bodies in PD and MPTP model and are often less amenable to neuroprotection (Liberatore et al., 1999; Wu et al., 2002). It is possible that the use of a higher dosage or continuous dosing of K560 may rescue MPTP-induced striatal dopaminergic nerve terminals loss.

Previous studies have suggested that HDAC inhibitors are promising potential neuroprotective agents worthy of further investigation for future treatment of PD. However, tremendous work needs to be done to fully decipher the mechanism of these inhibitors due to their multi-targets and the fact that they have been proven to be useful in treating malignancies. Our present findings provide supportive evidence that selective inhibition of HDAC1/2 results in protective effect in dopaminergic neurons and may be a novel target for PD therapy. The use of isoform-specific HDAC1/2 inhibitor provides better understanding on the role of HDAC1/2 isoforms and the potential neuroprotection following their inhibition in MPP⁺/MPTP-induced PD model. Our study may take this field further as this would help to

discover and refine the mechanistic underpinnings of these diseases for improved targeted, mechanism-based treatments by reducing toxicity observed following the use of pan-inhibitors. K560 may be a valuable target for the development of new therapies for PD aimed at attenuating further loss of dopaminergic neurons but the ideal therapeutic approach may require combination with other agents that can promote nerve fiber regrowth and mediate recovery of striatal dopaminergic innervation density. Also, rigorous safety and efficacy testing of this compound in nonhuman primates should be a pre-requisite before advancing to human clinical trials. Effect of HDAC inhibitor K560 should also be tested in α -synuclein transgenic models to determine the role of epigenetic in PD. Continued research on small molecule, selective HDAC inhibitors will advance understanding of how these may be best applied in clinical neurodegenerative diseases therapy.

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Figure and table legends

Figure 1: HDAC1/2 isoform specific inhibitor K560 protects differentiated SH-SY5Y cells against MPP⁺-induced cytotoxicity. SH-SY5Y cells were treated with culture medium containing MPP⁺ in the presence or absence of HDAC inhibitor at the concentration indicated for 48 hr. DMSO or sterilized water was used as control. (a) Cell death was determined by LDH assay. Cells treated with K560, but not TSA, SAHA and VPA, showed greater resistance against MPP⁺-induced cell death, comparable to that of MS-275. (b) Representative images of dual staining of active caspase 3 (red) and nuclear stain DAPI (blue) of control and K560 treated cells with or without MPP⁺ insult. (c) Quantification of percentage of active caspase 3-positive cells of total cell from ten random 10x magnification microscopic field of view showed a lower percentage of active caspase 3-positive cells of total cells in K560 treated cells compared to control. Data represent mean $\pm$ SEM from three independent experiments. * $p < 0.05$ for the comparison of control versus HDAC inhibitor-treated cells under the same treatment condition by one-way-ANOVA. (Scale bars, 50 μ m)

Figure 2: MPP⁺ increases expression and activation of HDAC2 while K560 suppresses HDAC2 activity in differentiated SH-SY5Y cells. (a) Chemical structure of K560 reveals a 4-ethyl-2,3-dioxopiperazine-1-carboxamide group as the surface recognition domain and a 2-aminobenzamide moiety that exerts HDAC1/2-selective inhibition. (b) Western blot and (c) densitometric quantification of histone H3 acetylation level. K560 treatment upregulated histone acetylation. (d) Western blot and (e) densitometric quantification of HDAC1 and 2 levels. (f) Adenovirus-mediated HDAC2 promoter assay with the results expressed as the ratio of firefly to Renilla luciferase activity (Fluc/Rluc). MPP⁺ induced enhanced HDAC2 transcriptional activity and protein expression. (g) Effect of K560 and MPP⁺ on HDAC2 deacetylation activity using HDAC-Glo2 assay with SAHA serving as control. K560 inhibits

while MPP⁺ augments HDAC2 enzyme activity. Data represent mean $\pm$ SEM from three independent experiments. * $p < 0.05$ for the comparison of control versus K560 treated cells under the same treatment condition and # $p < 0.05$ for the comparison of control versus MPP⁺-treated cells by one-way-ANOVA.

Figure 3: Apoptotic protein profiling in mock and K560 treated SH-SY5Y cells with or without MPP⁺ insult. SH-SY5Y cells were treated with 2 mM MPP⁺ in the presence or absence of 1 μ M K560 for 48 hr. Cell extracts were prepared and analyzed using human apoptosis antibody array kit. (a) Representative array membranes. Five out of a total of 35 apoptosis-related proteins with significant changes are marked with square boxes and labeled. (b) Densitometric analysis revealed sustained high level of XIAP, livin, caspase and reduced p53 phosphorylation in K560 treated cells upon MPP⁺ insult compared to respective controls. Intensity of signals was normalized to reference control and results are expressed as fold increases. Data represent mean $\pm$ SEM from three independent experiments. * $p < 0.05$ for the comparison of control versus K560 treated cells under the same treatment condition and # $p < 0.05$ for the comparison of control versus MPP⁺-treated cells by one-way-ANOVA.

Figure 4: Protective effect conferred by K560 is XIAP-dependent and can be abolished by XIAP suppression. SH-SY5Y cells were treated with 2 mM MPP⁺ in the presence or absence of 1 μ M K560 for 48 hr. Cell lysates were prepared, resolved by SDS-PAGE and subjected to western blot analyses. (a) Representative western blots of XIAP expression with β -actin as equal loading control. (b) Relative densitometric analyses of XIAP protein levels normalized to β -actin control. MPP⁺-induced reduction of XIAP was prevented by K560 addition. (c) Silencing of XIAP expression by LNA antisense oligonucleotides. Cells were transfected with 200 nM XIAP targeting LNA oligonucleotides with 5'-fluorescein-labeled

oligonucleotide serving as negative control. Twenty-four hour post transfection, cells were subjected to MPP⁺ and K560 treatments. Fluorescent microscopy of control cells transfected with fluorescein-labeled oligonucleotide revealed overall transfection efficiency was about 50%. (d) Western blot analysis confirmed the down-regulation of XIAP protein expression in cells transfected with XIAP-targeting LNA antisense oligonucleotide. (e) XIAP knockdown intensified MPP⁺-induced cell death and abolished the protective effect of K560 against MPP⁺ toxicity. Data represent mean $\pm$ SEM from three independent experiments. * $p < 0.05$ for the comparison of control versus K560 treated cells and # $p < 0.05$ for the comparison of control versus MPP⁺-treated cells or XIAP LNA transfected cells under the same treatment condition by one-way-ANOVA. (Scale bars, 25 μ m)

Figure 5: K560 mediates protection against MPP⁺ toxicity through down-regulation of PUMA and inactivation of Raf-MEK-ERK MAPK cascades. (a)(i) Representative western blots of p53 and PUMA expression with β -actin as equal loading control. Relative densitometric analyses of (ii) p53 and (iii) PUMA protein levels normalized to β -actin control. K560 impeded p53 phosphorylation without affecting its protein level and down-regulated PUMA. (b)(i) Representative western blots of phospho-SAPK/JNK expression with SAPK/JNK as equal loading control. (ii) Relative densitometric analyses of phospho-SAPK/JNK protein levels normalized to SAPK/JNK. K560 treated cells showed less phosphorylation of SAPK/JNK both at basal level and after MPP⁺ addition than that of control cells. (iii) Inhibition of JNK by SP60025 at 1 μ M did not suppress MPP⁺ induced cell death. (c)(i) Representative western blots of phospho-Raf, MEK and ERK expression with β -actin as equal loading control. Relative densitometric analyses of (ii) Raf, (iii) MEK and (iv) ERK protein levels normalized to β -actin control. K560 counteracted MPP⁺-induced phosphorylation of Raf, MEK and ERK1/2. (v) MEK inhibitor, U0126, at 5 μ M attenuated

MPP⁺ induced cell death. Data represent mean $\pm$ SEM from three independent experiments. * $p < 0.05$ for the comparison of control versus K560 or U0126 treated cells and # $p < 0.05$ for the comparison of control versus MPP⁺-treated cells under the same treatment condition by one-way-ANOVA.

Figure 6: K560 pre-treatment exerts protective effect against dopaminergic cell loss following subacute MPTP dosing regime in C57BL/6 mice. (a) Experimental schedule of K560 and MPTP treatment and sample collection. Oral administration of K560 at 45 mg/kg started two days before the first MPTP injection and lasted for seven consecutive days. MPTP was administered intraperitoneally at 30 mg/kg/day for five consecutive days. Western blotting and TH cell count were conducted on day 2 and 21 after last MPTP injection. (b) Blood brain barrier permeability of K560 was validated by LC-MS/MS assay and western blot analysis showed increased level of histone H3 acetylation in brains of administered mice, with MS275 as reference compound. (c) Stereological count of TH- and Nissl-double-positive dopaminergic neuron of SNpc 2 days and 21 days following subacute MPTP administration. (d) Photomicrograph representative of the state of the nigral dopaminergic system following MPTP and K560 treatments. (e) Representative western blots and (f) densitometric analysis of TH and XIAP expression levels in SNpc 2 days and 21 days following subacute MPTP administration. K560 pre-treatment attenuates the dopaminergic neurotoxicity induced by MPTP with sustained XIAP. Data represent mean $\pm$ SEM. * $p < 0.05$ for the comparison of none/MPTP- versus K560/MPTP-treated group and # $p < 0.05$ for comparison of none/saline- versus none/MPTP-treated group by one-way-ANOVA; $n \geq 3$ mice per group. (Scale bars, 100 μ m)

Supplementary data

Figure 1: Selective HDAC3 inhibition may not be ideal target for neuroprotection in experimental PD. (a) Effect of HDAC3 selective inhibitor, compound 11 against MPP⁺ toxicity. (b) Effect of HDAC2 and 3 selective inhibitor, apicidin, against MPP⁺ toxicity. Both compound 11 and apicidin showed no protective effect. Data represent mean $\pm$ SEM from three independent experiments. * $p < 0.05$ for the comparison of control versus HDAC inhibitor-treated cells under the same treatment condition by one-way-ANOVA.

Figure 2: Striatal nerve fibers are not rescued from MPTP neurotoxicity by K560 treatment. (a) Photomicrograph representative of TH staining in striatum 2 days following subacute MPTP administration with or without K560 oral administration. (b) Densitometric analysis of striatal TH fiber density using Image J software. (c) Representative western blots and (d) densitometric analysis of TH expression levels in striatum. Data represent mean $\pm$ SEM. # $p < 0.05$ for comparison of none/saline- versus none/MPTP-treated group by one-way-ANOVA; $n \geq 3$ per mice group. (Scale bars, 200 μ m)

Figure 3: K560 post-treatment mitigates MPTP-induced SNpc dopaminergic cell loss in C57BL/6 mice. (a) Experimental schedule of K560 and MPTP treatment and sample collection. MPTP was administered intraperitoneally at 30 mg/kg/day for five consecutive days. Oral administration of K560 at 45 mg/kg started after the day of last MPTP injection and lasted for seven consecutive days. Western blotting and TH cell count were conducted on day 21 after last MPTP injection. (b) Stereological count of TH- and Nissl-double-positive dopaminergic neuron of SNpc 21 days following subacute MPTP administration. (c) Representative western blots and (d) densitometric analysis of TH expression levels in SNpc 21 days following subacute MPTP administration. K560 post-treatment attenuates the

dopaminergic neurotoxicity induced by MPTP. Data represent mean $\pm$ SEM. * $p < 0.05$ for the comparison of none/MPTP- versus K560/MPTP-treated group and # $p < 0.05$ for comparison of none/saline- versus none/MPTP-treated group by one-way-ANOVA; $n \geq 3$ mice per group.

Table 1: Classification of HDAC inhibitors

Figure(s)

Figure 1

Figure 2

Figure 3

a

b

Figure 4

a

b

c

d

e

Figure 5

a

b

c

Figure 6