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Targeting cyclin D3/CDK6 activity for treatment of Parkinson's disease.

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List of Abbreviations used:

6-OHDA: 6-hydroxy-dopamine

AD: Alzheimer's disease

BBB: Blood brain barrier

CDK6: Cyclin-dependent kinase 6

DMEM: Dulbecco's Modified Eagle Medium

EBV: Epstein-Barr virus

ECL: Enhanced chemiluminiscence

FBS: Fetal bovine serum

FTLD: Frontotemporal lobar degeneration

HDAC: Histone deacetylase

LCLs: Lymphoblastoid cell lines

m-TOR: Mammalian target of rapamycinm

MTT: (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

NaB: Sodium butyrate

PBMCs: Peripheral Blood Mononuclear Cells

PBS: Phosphate buffered saline

PD: Parkinson's disease

PI: Propidium iodide

pRb: Retinoblastoma protein

PVDF: Polyvinylidene difluoride

SAHA: Suberoylanilide hydroxamic acid

SDS-PAGE: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

UPDRS: Unified Parkinson's Disease Rating Scale

UPS: Ubiquitin proteasome system

ABSTRACT

At present, treatment for Parkinson's disease (PD) is only symptomatic, therefore it is important to identify new targets tackling the molecular causes of the disease. We previously found that lymphoblasts from sporadic PD patients display increased activity of the cyclin D3/CDK6/pRb pathway and higher proliferation than control cells. These features were considered systemic manifestations of the disease, as aberrant activation of cell cycle is involved in neuronal apoptosis. The main goal of this work was to elucidate whether the inhibition of cyclin D3/CDK6-associated kinase activity could be useful in PD treatment. For this purpose, we investigated the effects of two histone deacetylase (HDAC) inhibitors, suberoylanilide hydroxamic acid (SAHA) and sodium butyrate (NaB), and the m-TOR inhibitor rapamycin on cell viability and cyclin D3/CDK6 activity. Moreover, the potential neuroprotective action of these drugs was evaluated in 6-hydroxy-dopamine (6-OHDA) treated dopaminergic SH-SY5Y cells and primary rat mesencephalic cultures. Here we report that both compounds normalized the proliferative activity of PD lymphoblasts and reduced the 6-OHDA-induced cell death in neuronal cells by preventing the overactivation of the cyclin D3/CDK6/pRb cascade. Considering that these drugs are already used in clinic for treatment of other diseases with good tolerance, it is plausible that they may serve as novel therapeutic drugs for PD.

INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disorder characterized by the progressive loss of dopaminergic neurons in the substantia nigra of the mid-brain. The clinical characteristics of PD are disabling motor abnormalities, including tremor, muscle rigidity, paucity of voluntary movements, and postural instability (Jankovic 2008). Other non-motor features like mood disorders, sleep disturbances, and cognitive impairment are also associated to PD (Chaudhuri & Schapira 2009). Approximately 90% of cases of PD are considered sporadic, being the rest determined by gene mutations. It is believed that non-genetic factors could play an important role in sporadic PD, by interacting with susceptibility genes. Age is a major risk factor for idiopathic PD. The average age at onset is 60 years and disease duration is approximately 15 years (Fan *et al.* 2013, Samii *et al.* 2004).

Current treatment strategies for PD include symptomatic treatments that consist primarily on replacing dopamine in the brain with levodopa or dopamine agonists (Djamshidian *et al.* 2014, Pahwa & Lyons 2014). Nevertheless, the chronic dopamine replacement therapy can cause debilitating side effects as bradykinesia. Consequently, it is important to develop new neuroprotective strategies that can help to halt or slow the progression of PD.

The cause of neuronal death in PD and other neurodegenerative disorders is largely unknown, but it appears that apoptosis is the final fate of vulnerable neurons in PD (Hajimohamadreza & Treherne 1997). Mounting evidence suggest that neuronal apoptosis is the result, at least in part, of dysregulation of cell cycle control in post-mitotic neurons (Copani *et al.* 2001). First of all, cell cycle-related proteins have been shown to be elevated in neurons that are at risk to die in various neurodegenerative disorders including PD (Jordan-Sciutto *et al.* 2003). Second, some cell cycle inhibitors appear to have neuroprotective properties in cell culture and animal models (Alvira *et al.* 2007, Park *et al.* 2000).

Work carried out in the last decade in our laboratory demonstrated the existence of cell cycle disturbances in immortalized lymphocytes from Alzheimer's disease (AD), frontotemporal lobar degeneration (FTLD) and PD (Alquezar *et al.* 2012, Bartolome *et al.* 2009, Esteras *et al.* 2014). Lymphoblasts from sporadic PD were shown to have a higher proliferative activity than cells from control individuals associated with a cyclin D3/CDK6-dependent hyperphosphorylation of pRb (Esteras et al. 2014). Considering that CDKs and/or cyclins have been reported in a number of in vitro neuronal death paradigms (Katchanov *et al.* 2001, Osuga *et al.* 2000, Rashidian *et al.* 2005), we think that lymphoblasts, easily accessible cells, from sporadic PD patients could be a useful model to study cell cycle-related events in PD pathology, and a suitable platform to test the effects of drugs that have been proven to be useful in other diseases.

In particular, this work was undertaken to explore whether treatment with histone deacetylase (HDAC) inhibitors like sodium butyrate (NaB) or vorinostat, suberoylanilide hydroxamic acid (SAHA), which inhibit CDK6 expression levels, as well as the immunosuppressant rapamycin that downregulates cyclin D3 content, could normalize the proliferative response of PD lymphoblasts. Moreover, the potential neuroprotective action of these drugs was evaluated in well-established cell models for PD such as the 6-hydroxy-dopamine (6-OHDA)-treated dopaminergic SH-SY5Y cells and 6-OHDA-treated primary rat mesencephalic cultures.

The results herein reported suggest that cyclin D3/CDK6 could be considered as a novel pharmacological target for PD, and that the repositioning of SAHA, NaB and rapamycin, drugs already licensed for different conditions, towards the treatment of PD could be a time- and cost-saving strategy.

MATERIALS AND METHODS

Materials

All components for cell culture were obtained from Invitrogen (Carlsbad, CA). L-DOPA, 6-OHDA, the HDAC inhibitors, NaB and SAHA, and rapamycin were obtained from Sigma-Aldrich. PVDF (polyvinylidene difluoride) Immobilon-P membranes for Western blots were purchased from Millipore. Rabbit anti-human antibodies, such as pRb (sc-50), CDK6 (sc-177), and mouse anti-human antibodies α-tubulin (sc-23948) and β-actin (sc-47778) were from Santa Cruz Biotechnologies. Mouse anti-human Cyclin D3 was obtained from Cell Signaling. Mouse anti-human Lamin B was from Calbiochem. The enhanced chemiluminiscence (ECL) system was from Amersham. All other reagents were of molecular grade.

Cell lines

A total of 20 patients were diagnosed in the department of Neurology of the University Hospital Doce de Octubre (Madrid, Spain) of sporadic PD following Diagnostic Criteria published elsewhere (Gelb *et al.* 1999). All patients were undergoing treatment with levodopa, alone or in combination with dopamine agonists (13 cases), selegiline or rasagiline (2 cases). A group of 20 age-matched individuals was used as control. The control group was formed of family members or caregivers of the PD patients, and showed a completely normal cognitive and functional level. Demographic characteristics of PD patients and control subjects are presented in Table 1. In all cases peripheral blood samples were obtained after written informed consent of the donors. All study protocols were approved by the Spanish Council of Higher Research Institutional Review Board and are in accordance with National and European Union Guidelines. Peripheral Blood Mononuclear Cells (PBMCs) were isolated on Lymphoprep™ density-gradient centrifugation according to the instructions of the manufacturer (Axix-Shield). Cells were washed twice with phosphate buffered saline (PBS), counted and resuspended at the desired concentration.

Establishment of lymphoblastoid cell lines (LCLs) was performed in our laboratory as previously described (Ibarreta *et al.* 1997) by infecting PBMCs with the Epstein-Barr virus (EBV). Cells were grown in suspension in T flasks in an upright position, in approximately 10 mL RPMI 1640 (Gibco, BRL) medium that contained 2 mM L-glutamine, 100 μg/mL penicillin/streptomycin and, unless otherwise stated, 10% (v/v) fetal bovine serum (FBS) and maintained in a humidified 5% CO_2 incubator at 37 ºC. Fluid was routinely changed every 3 days by removing the medium above the settled cells and replacing it with an equal volume of fresh medium.

Neuronal cell culture

Human neuroblastoma (SH-SY5Y) cells were purchased from the European Collection of Cell Cultures (Health Protection Agency, Salisbury, UK), and were propagated in Dulbecco's Modified Eagle Medium (DMEM) containing L-glutamine (2 mM), 1% nonessential amino acids, 1% penicillin/streptomycin and 10% fetal bovine serum (FBS) under humidified 5% CO2 and 95% air. On attaining semiconfluence, cells were treated with 6-OHDA (35 μM) for 24 hours. Some cultures were pretreated for 1 hour with different compounds at several concentrations. After treatment, cultures were processed for cell viability assay or Western blotting.

Primary ventral mesencephalic cell culture

Young (3-5 months) Wistar rats were obtained from the inbred colony of the Research Institute, Hospital Doce de Octubre, Madrid, Spain. Primary mesencephalic cultures were prepared from rat embryos (E15-16) according to methods described previously with some modification (Yin *et al.* 2012, Pruszak *et al.* 2009). 1 x 10^5 cells were plated in pretreated poly-D-lysine-coated 96-well plates. Cells were maintained at 37ºC in a 5% CO_2 humidified atmosphere in Neurobasal A medium (Gibco) that contained 2 mM L-glutamine, and 10% B27® Supplement (Invitrogen). 9 days after cell culture preparation, mesenchephalic cells were treated with 35 μM of 6-OHDA for 24 hours and pretreated or not for 1 hour with increasing concentrations of SAHA, NaB and rapamycin. After treatment, cultures were processed for cell viability assay (MTT). All animals were handled and cared for Council Directive 2010/63/UE of September 22, 2010.

Measurement of cell proliferation, cell viability and cell cycle

Cell proliferation was determined by total cell counting, using a TC10™ Automated Cell Counter (Bio-Rad Laboratories, Madrid, Spain). Cells failing to exclude the dye were considered nonviable. Cell viability was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, as previously described (Denizot & Lang 1986). Cell survival was estimated as the percentage of the value of untreated controls. Cell cycle phase distribution was routinely determined by cell permeabilization followed by propidium iodide (PI) staining and flow cytometry analysis using an EPICS-XL cytofluorimeter (Coulter Científica, Móstoles, Spain).

Immunoblotting analysis

Whole-cell extract preparation, SDS-PAGE and Western blot analysis of lymphoblasts or neuroblastoma cells were performed as described in previous publications (Alquezar et al. 2012, Esteras et al. 2014). Primary antibodies were used at the following dilutions: 1:500 anti-pRb, 1:500 anti-cyclin D3, 1:1000 anti-CDK6, 1:1000 anti-β-actin, 1:1000 anti-α-tubulin, 1:1000 anti-Lamin B. Protein band densities were quantified using Image J software (NIH, Bethesda, Maryland, USA) after scanning the images with a GS-800 densitometer from BioRad.

Quantitative Real-Time PCR

Total RNA was extracted from cell cultures using Trizol™ reagent (Invitrogen). RNA yields were quantified spectrophotometrically and RNA quality was checked by the A260/A280 ratio and on a 1.2% agarose gel to observe the integrity of 18S and 28S rRNA. RNA was then treated with DNase I Amplification Grade (Invitrogen). One microgram was reverse transcribed with the Superscript III Reverse Transcriptase kit (Invitrogen). Quantitative real-time PCR was performed in triplicates using TaqMan™ Universal PCR MasterMix No Amperase UNG (Applied Biosystems) reagent according to the manufacturer's protocol. Primers were designed using the Universal Probe Library for Human (Roche Applied Science) and used at a final concentration of 20 μM. The sequences of the forward and reverse primers used are 5'-tgatcaactaggaaaaatcttggac-3' and 5'-ggcaacatctctaggccagt-3' for CDK6 and 5'-ccaaccgcgagaagatga-3' and 5'-ccagaggcgtacagggatag-3' for β-actin.

Real time quantitative PCR was performed in the Bio-Rad iQ5 system using a thermal profile of an initial 5-min melting step at 95ºC followed by 40 cycles at 95ºC for 10s and 60ºC for 60s.

Relative mRNA levels of the genes of interest were normalized to β-actin expression using the simplified comparative threshold cycle delta *CT* method [$2^{(\Delta CT\ CDK6-\Delta CT\ \beta\text{-}actin}$].

Statistics

Statistical significance was determined by the Student's *t*-test or two-way analysis of variance (ANOVA) followed by Bonferroni's test for multiple comparisons using GraphPad Prism 6 for Macintosh (La Jolla, CA, USA). A value of $p<0.05$ was considered significant. All the statistical data are presented as mean ± standard error of the mean (SEM).

RESULTS

Proliferative activity of control and PD lymphoblasts

Data in Fig. 1A shows proliferation of all cell lines derived from PD patients and age-matched control individuals used in this study. For these experiments, cells were seeded at an initial density of 1 x 10^6 cells x mL^{-1} in presence of 10% FBS and counted 72 hours after serum stimulation. In agreement with previous reports (Esteras et al. 2014), lymphoblasts from PD patients, showed higher rates of cell proliferation than those of control cells. To rule out an adverse effect of levodopa treatment to PD patients that could persist the immortalization procedure, we incubated a small number of lymphoblastoid cell lines from control and PD patients with L-DOPA. As shown in Fig. 1B, L-DOPA had no effect on proliferation of control and PD cells. As the enhanced proliferative activity of PD lymphoblasts was associated to the cyclin D3/CDK6-induced phosphorylation of the retinoblastoma protein (pRb) (Esteras et al. 2014), we also investigated the effect of L-DOPA addition in the cellular levels of these proteins. Fig. 1C shows, as expected, increased levels of phosphorylated pRb, CDK6, and cyclin D3 proteins of PD lymphoblasts. In addition it is shown that L-DOPA did not modify the cellular content of these proteins.

Effects of histone deacetylase inhibitors and rapamycin on cell proliferation of control and PD lymphoblasts

Bearing in mind that CDK6 has shown to be epigenetically regulated by histone deacetylases (HDAC) (Kim *et al.* 2007, Giessrigl *et al.* 2013), we considered interesting to evaluate the effects of two different HDAC inhibitors, sodium butyrate (NaB) and suberoylanilide hydroxamic acid (SAHA) on the cell response to serum stimulation. SAHA is a US Food and Drug Administration-approved drug for the treatment of cutaneous T-Lymphoma (Slingerland *et al.* 2014) and NaB is being evaluated in clinical trials for the treatment of shigellosis (Raqib *et al.* 2012) and it is thought that could be effective on the treatment of colorectal cancer (Goncalves & Martel 2013). In addition and since degradation of cyclin D3 seems to be impaired in PD lymphoblasts (Esteras et al. 2014), we investigated the influence of rapamycin, a US Food and Drug Administration-approved drug used as immunosuppressant (McAlister *et al.* 2002), in the proliferative response of control and PD lymphoblasts. Rapamycin was shown to downregulate cyclin D3 levels by increasing its degradation in the ubiquitin proteasome system (UPS) (Hleb *et al.* 2004).

To investigate the effects of these drugs on cell proliferation, lymphoblasts were incubated in the absence or in the presence of SAHA, NaB or rapamycin for 72 hours and cell viability was determined by the MTT assay. Treatment with escalating concentrations (0-2 μM) of SAHA reduced the cell viability in a dose-dependent manner (Fig. 2A left panel). Similar results were

obtained with increasing concentrations of NaB up to 50 μM (Fig. 2B left panel) or with increasing concentrations of rapamycin (Fig. 2C, left panel). Maximal effects of SAHA, NaB and rapamycin were obtained at 1 μM, 10 μM, and 30 ng/mL respectively. The proliferative activity of control and PD lymphoblasts was further assessed by total cell counting after treating cells with SAHA, NaB, or rapamycin for 72 hours. As shown in Fig. 2A-C right panels, the addition of SAHA (1 μM), NaB (10 μM) or rapamycin (30 ng/mL) abrogated the enhanced proliferative response of cells from PD patients, affecting in a lesser extent the proliferation of control cells.

Effects of SAHA, NaB and rapamycin on cell cycle status of control and PD lymphoblasts

Since the change in cell number depends on the balance between cell proliferation and cell death, we tested if SAHA, NaB or rapamycin treatment induced cell death by necrosis/apoptosis or by contrast, were able to decrease the enhanced cell proliferation observed in PD lymphoblasts. For this purpose, lymphoblasts from control and PD individuals were stained with propidium iodide (PI) and the distribution of cells in the cell cycle phases was analyzed by flow cytometry. We did not observe significant changes in the proportion of sub-G_0/G_1 hypodiploid cells, characteristic of apoptosis/necrosis, in control and PD lymphoblasts after SAHA, NaB or rapamycin treatment (Fig. 3). SAHA was more effective in decreasing the percentage of cells in S/G_2M phases than the other HDAC inhibitor NaB. Taken together, these results suggest that the decreased cell number in cultures of PD lymphoblasts in the presence of all the drugs used in this work truly reflects a decrease in cell proliferation.

Effects of SAHA, NaB and rapamycin on cyclin D3/CDK6 levels and associated kinase activity

We next evaluated the impact of SAHA, NaB and rapamycin treatment on the levels of cyclin D3 and CDK6 proteins and in their associated kinase activity, assessed indirectly by the phosphorylation status of pRb, as well as in the levels of *CDK6* mRNA. In agreement with our previous work (Esteras et al. 2014), we found that the relative *CDK6* mRNA levels were elevated in PD cells compared with those of control cells (Fig. 4A). As expected, both HDAC inhibitors were able to blunt the increase in *CDK6* expression. At the dose used (1 μM SAHA or 10 μM NaB), they had no effect in *CDK6* mRNA levels in control cells (Fig. 4A). In consonance with these results, Fig. 4B shows that these compounds were effective in restoring the normal levels of CDK6 protein and in preventing the increased phosphorylation of pRb in PD lymphoblasts. The effect of 30 ng/mL of rapamycin in decreasing the levels of cyclin D3 in control and PD lymphoblasts is shown in Fig. 4C (left panel). Consequently, the addition of rapamycin partially prevented the higher phosphorylation of the pRb protein in lymphoblasts derived from PD patients (Fig. 4C right panel).

Neuroprotective effect of SAHA, NaB and rapamycin on 6-OHDA-induced death in neuroblastoma SH-SY5Y cells and dopaminergic primary cultures

We considered interesting to validate the above-described results in lymphoblastoid cell lines by testing the effects of cyclin D3/CDK6 kinase activity-modifying drugs in two well-established neuronal models of PD, neuroblastoma SH-SY5Y cells (Ouyang & Shen 2006) and primary cultures of dopaminergic mid-brain cells (Pruszak et al. 2009). To this end, cells were pretreated for 1 hour with SAHA, NaB, or rapamycin before 6-OHDA (35 μM) exposure. Fig. 5 summarizes the effects of these drugs on 6-OHDA-treated SH-SY5Y neuroblastoma cells at the same dose used in the experiments with lymphoblasts. Fig. 5A shows that, as expected, the addition of 6-OHDA to the SH-SY5Y cells resulted in significant cell death (50%). The pre-treatment of these cells with the HDAC inhibitors or rapamycin partially prevented the cell death induced by the toxin 6-OHDA. To elucidate whether changes in cyclin D3/CDK6 activity are involved in the cell death induced by this neurotoxin, we determined the levels of both CDK6 and cyclin D3 as well as the phosphorylation status of the pRb protein in the absence and in the presence of SAHA, NaB and rapamycin. 6-OHDA induced significant increases in CDK6 and cyclin D3 proteins (Fig. 5B) and therefore in the phosphorylated form of the pRb protein (Fig. 5C). The pretreatment with SAHA, NaB or rapamycin limited the increase in these proteins caused by the addition of 6-OHDA, suggesting the involvement of cyclin D3/CDK6 in neuronal survival.

Finally, we sought to evaluate the potential neuroprotective role of these drugs in non-dividing, post-mitotic neurons as primary rat mesencephalic neuronal cultures. To this end, cells were pretreated with escalating concentrations of SAHA (0-2 μM), NaB (0- 50 μM) or rapamycin (0-100 ng/mL) for 1 hour before 6-OHDA (35 μM) exposure, and cell viability was analyzed 24 hours later by MTT assay. Fig. 6 shows that the addition of 6-OHDA to the mesencephalic cells resulted in a significant cell death (40%). The pre-treatment of these cells with increasing concentrations of HDAC inhibitors (Fig. 6A) or rapamycin (Fig. 6B) partially prevented the cell death induced by the toxin 6-OHDA.

DISCUSSION

In this work, we have evaluated the effects of targeting cyclin D3/CDK6 activity on survival/death in cellular models of PD, particularly immortalized lymphocytes from sporadic PD patients, human dopaminergic SH-SY5Y neuroblastoma cells, and primary rat mid-brain cell cultures treated with 6-OHDA.

The use of peripheral cells form patients as surrogate tissue for allowing early diagnosis and more efficacious treatment for neurodegenerative disorders is widely accepted. Assuming that biomolecular defects associated with PD could be detected in any cell type, numerous studies have been carried out using peripheral cells isolated from blood (i.e. lymphocytes, monocytes, platelets, red blood cells) of PD patients (Irrcher *et al.* 2010, Saito *et al.* 2009, Zhong *et al.* 2006, Jakubauskiene *et al.* 2012). Moreover PBMCs can synthesize endogenous dopamine and express DA receptors (DA-Rs) and DA transporters (McKenna *et al.* 2002). Lymphoblastoid cell lines from patients can be easily obtained by infecting PBMCs with EBV. They resemble activated B cells in the expression of activation markers and adhesion molecules (Wang *et al.* 1990). Previous works from our laboratory demonstrated that EBV-immortalized lymphocytes respond to serum addition or withdrawal as fresh isolated lymphocytes do, and display similar changes in the content of certain proteins implicated in the regulation of proliferation and survival (Bartolome et al. 2009, Munoz *et al.* 2008). Taken together, these findings support the use of these lymphoblastoid cell lines for the study of disease and therapy-related modifications within *ex vivo* non-neural DA cells of individuals with PD. On the other hand, the human dopaminergic SH-SY5Y cell line and primary cultures of rat mid-brain neurons exposed to 6-OHDA are used commonly as *in vitro* models for PD, as it reflects cellular processes that occur in the degenerating dopaminergic neurons (Ouyang & Shen 2006, Kim *et al.* 2010).

Here, we confirmed previous data from our laboratory (Esteras et al. 2014) indicating a higher rate of proliferation of PD lymphoblasts than cells from age-matched control individuals, that was associated with increased cyclin D3/CDK6-mediated phosphorylation of the pRb protein. Activation of the pRb pathway has been reported in PD brain and in mouse models of PD (Hoglinger *et al.* 2007) although in neurons this leads to cell death rather than to cell division. Thus, our results support the hypothesis that unscheduled cell cycle activation underlies neurodegeneration.

Since positive regulation of CDK6 expression is achieved by HDACs (Dhar *et al.* 2014) and considering that inhibitors of HDACs have been theorized to be as efficacious in neurodegenerative diseases as in cancer (Harrison & Dexter 2013), we investigated whether two different HDAC inhibitors, SAHA and NaB, which are known to cross the blood brain barrier (BBB) (Barichello *et al.* 2014, Palmieri *et al.* 2009) could normalize the cell response of PD lymphoblasts. Although SAHA and NaB differ in their chemical structure (hydroxamate, or

short chain fatty acid), they both inhibited the Zn-dependent HDACs, mainly of the class I and II. SAHA and NaB were effective in decreasing the serum-mediated increase of CDK6 levels and restored the normal rate of cell proliferation in PD lymphoblasts. These results are in line with a previous report indicating that these two broad classes of HDAC inhibitors (I and II) decreased proliferation of neural stem cells by blocking G_1/S transition (Zhou *et al.* 2011), as well as in other normal or transformed cells (Riester *et al.* 2007). The concentration of SAHA (1 μM) falls within the range of the peak serum concentration achieved in patients treated with effective oral anti-tumor doses of this compound (Kelly *et al.* 2005). Moreover, the fact that SAHA was shown to induce changes in inflammatory markers in a mouse model of septic shock (Finkelstein *et al.* 2010) suggest that SAHA may have secondary beneficial effects in PD besides normalizing CDK6 levels. On the other hand, NaB has been reported to reduce degeneration of dopaminergic neurons in a mutant alpha-synuclein drosophila transgenic model of familial PD (Kontopoulos *et al.* 2006) and to rescue the rotenone-induced locomotor impairment and early mortality in flies (St Laurent *et al.* 2013), suggesting a possible benefit in the early step of PD.

Rapamycin or sirolimus is widely used to prevent transplant rejection, due to its good efficacy and reduced toxicity (Morath *et al.* 2007). Moreover, rapamycin has been shown to effectively inhibit the growth of several murine and human cancer (Vignot *et al.* 2005). The capacity of rapamycin to stimulate autophagy makes this dug attractive for neurodegenerative disorders associated with impaired organelle and protein turnover (Pignataro *et al.* 2011). Our results show an antiproliferative effect of rapamycin in PD lymphoblasts, which can be explained by the effects of rapamycin restoring both the increased cyclin D3 levels and its associated kinase activity.

Although PD-associated changes detected in peripheral cells from patients might not fully reflect those of PD brain, it is worth to mention that at the doses used in this work, NaB, SAHA and rapamycin were able to partially prevent the cell death induced by 6-OHDA in neuronal models of PD, as the human neuroblastoma SH-SY5Y cell line or primary rat mid-brain dopaminergic neuronal cultures. These results are in agreement with previous work reporting the effects of this neurotoxin in pRb phosphorylation (Rodriguez-Blanco *et al.* 2008), and add further support to the hypothesis that cell cycle alterations underlie neuronal loss. Taking together it seems that lymphoblasts from PD patients can be considered as a useful experimental model to study molecular events associated with the disease, as well as an interesting platform for testing novel therapies.

In conclusion, our data indicate that increased proliferation of PD lymphoblasts is causally linked to overactivation of cyclin D3/CDK6 complex, and that treatments aimed at blocking cyclin D3/CDK6-associated kinase activity are effective in decreasing the enhanced cell proliferation of PD lymphoblasts. The proposed scenario is represented schematically in Figure

7. NaB and SAHA, by inhibiting CDK6 protein expression, or rapamycin by decreasing the cellular levels of cyclin D3, are able to rescue the normal rates of proliferation of PD lymphoblasts.

Considering that the HDAC inhibitors as well as rapamycin are already drugs used for the treatment of several diseases with a good tolerance it seems reasonable to believe that they may hold promise as a novel treatment for PD. The repositioning of these known drugs towards PD may be therefore a time and cost-saving strategy.

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DISCLOSURE

None of the authors has any conflict to disclose

AUTHOR'S CONTRIBUTIONS

AM-R conceived and designed the experiments. JAM recruited and diagnosed the PD patients. . FB generated the lymphoblastoid cell lines and helped with the neuronal cultures. CA, EB, NE and AdlE carried out the experimental work, analyzed the data, prepared the figures and performed the statistical analysis. AM-R and CA wrote the manuscript. All authors read and approved the final manuscript.

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TABLE 1.
Demographic characteristics of PD patients and healthy subjects

	Control	Parkinson's Disease
Number	20	20
Gender (M/F)	4/16	16/4
Age study	63.0 ± 2.8	69.2 ± 2.2
Age onset	NA	64.5 ± 2.2
Hoehn and Yahr scale	NA	2.3 ± 0.2
UPDRS	NA	24.8 ± 1.6
Levodopa dosage (mg/24hours)	NA	603.3 ± 62.7

Values are expressed as mean ± SEM. NA not applied

FIGURE LEGENDS

Figure 1

Proliferation of lymphoblasts from control and PD patients

Control and PD lymphoblasts were seeded at an initial density of 1 x 10^6 cells x mL^{-1} and synchronized by serum starvation for 24 hours. On that point, 10% FBS was added alone (**A**) or in combination with L-DOPA (100 μM) (**B**) and 72 hours later samples were taken and counted using a TC10^{TM} Automated Cell Counter from BioRad. **C.** For Western blot analysis, cells were harvested 24 hours after L-DOPA addition and whole-cell lysates were prepared to analyze the levels of pRb, CDK6, and cyclin D3 proteins. Representative immunoblots are shown. pp = the hyperphosphorylated form of the pRb protein. Values shown represent the mean ± SEM of the quantification of the corresponding band in six independent observations carried out with different cell lines. (* $p<0.05$ and ** $p<0.01$ significantly different from control cells).

Figure 2

Effects of HDAC inhibitors and rapamycin on the proliferation of lymphoblasts from control and PD patients

Immortalized lymphocytes from control and PD patients, were seeded at an initial density of 1 x 10^6 x mL^{-1} and were incubated in medium containing 10% FBS, in the absence or in presence of increasing doses of SAHA (0-2 μM) **(A left panel)**, NaB (0-50 μM) **(B left panel)** and rapamycin (0-100 ng/mL) **(C left panel)** for 72 hours. A total of 100,000 cells per well were seeded in a 96-well plaque for the MTT assay. Results represent the % of cell proliferation of treated cells referred to untreated ones. **A, B, and C right panels.** Effect of the treatment with SAHA (1 μM), NaB (10 μM), or rapamycin (30 ng/mL) on proliferation of control and PD lymphoblasts. Aliquots were taken for cell counting 72 hours after the drug administration. Data shown are the mean ± SEM of ten determinations for each drug carried out with different cell lines (* $p<0.05$ and ** $p<0.01$ significantly different from control cells. †$p<0.05$ and ††$p<0.01$ significantly different from untreated cells).

Figure 3

Cell cycle distribution of lymphoblasts from control and PD patients after treatment with HDAC inhibitors or rapamycin

Immortalized lymphocytes from control and PD patients were seeded at an initial density of 1 x 10^6 x mL^{-1} and cultured in RPMI medium containing 10% FBS in the absence or in the presence of the HDAC inhibitors SAHA (1 μM) and NaB (10 μM) or rapamycin (30 ng/mL). 24 hours after drugs addition, cells were harvested, fixed, and analyzed by flow cytometry as described under Methods. The percentage of cells in the different cell cycle phases is indicated for each condition**.**

Figure 4

Effects of SAHA, NaB and rapamycin on cyclin D3/CDK6 levels and pRb phosphorylation status

Immortalized lymphocytes from control and PD patients were seeded at an initial density of 1 x 10^6 x mL^{-1}, and were incubated in RPMI medium containing 10% FBS in the absence or in the presence of SAHA (1 μM), NaB (10 μM) or rapamycin (30 ng/mL). 24 hours after treatment cells were harvested to analyze *CDK6* mRNA expression by quantitative RT-PCR or the levels of cyclin D3, CDK6 and pRb proteins by Western blotting. **A.** *CDK6* mRNA expression levels after SAHA and NaB treatment. Data shown represent the mean ± SEM of different experiments carried out with five control and five PD patients. **B and C.** Representative immunoblots showing the effect of SAHA and NaB on CDK6 and pRb proteins or the effect of rapamycin on cyclin D3 levels and pRb phosphorylation status. pp = the hyperphosphorylated form of the pRb protein. The densitometric data represent the mean ± SEM of different experiments using all the individuals enrolled in this study (*$p<0.05$ and **$p<0.01$ significantly different from control cells; †$p<0.05$ and ††$p<0.01$ significantly different from untreated cells).

Figure 5

Effects of HDAC inhibitors and rapamycin on survival and protein levels of pre-treated 6-OHDA SH-SY5Y neuroblastoma cells.

Neuroblastoma SH-SY5Y cells were exposed to 35 μM 6-OHDA for 24 hours in the presence or in the absence of HDAC inhibitors (1 μM for SAHA and 10 μM for NaB) or rapamycin (30 ng/mL). **A**. Number of viable cells after drug treatments measured by MTT assay. Each data point represents the mean ± SEM of three replications in four different experiments. (**p< 0.01 significantly different from SH-SY5Y untreated cells; †p<0.05 and ††p<0.01 significantly different from 6-OHDA-treated cells). **B and C**. CDK6, cyclin D3 and pRb protein levels assessed by Western blotting. Representative immunoblots are shown. The densitometric data represent the mean ± SEM of four different experiments (*p<0.05 and **p<0.01 significantly different from SH-SY5Y untreated cells; †p<0.05 and ††p<0.01 significantly different from 6-OHDA-treated cells). pp = the hyperphosphorylated form of the pRb protein.

Figure 6

Neuroprotective effects of HDAC inhibitors and rapamycin in 6-OHDA pre-treated mesencephalic primary cultures

A and B. Primary cultures of rat mid-brain cells were exposed to 35 μM 6-OHDA for 24 hours in the presence or in the absence of different concentrations of HDAC inhibitors (0-2 μM for SAHA and 0-50 μM for NaB) (**A**) or rapamycin (0-100 ng/mL) (**B**). The number of viable cells was measured by MTT assay. Each data point represents the mean ± SEM of four replications in four different experiments. (**p< 0.01 significantly different from untreated cells; †p<0.05 and ††p<0.01 significantly different from 6-OHDA-treated cells).

Figure 7

Diagram summarizing the effects of targeting the cyclin D3/CDK6 complex in the proliferative activity of Parkinson's disease lymphoblasts

Lymphoblasts from PD patients show an overactivation of the cyclin D3/CDK6-associated kinase activity compared with that of control cells. Treatment of PD cells with the HDAC inhibitors NaB and SAHA or targeting the cyclin D3 degradation by rapamycin normalized the cellular content of the cyclin D3/CDK6 complex, and restored normal rates of cell proliferation.

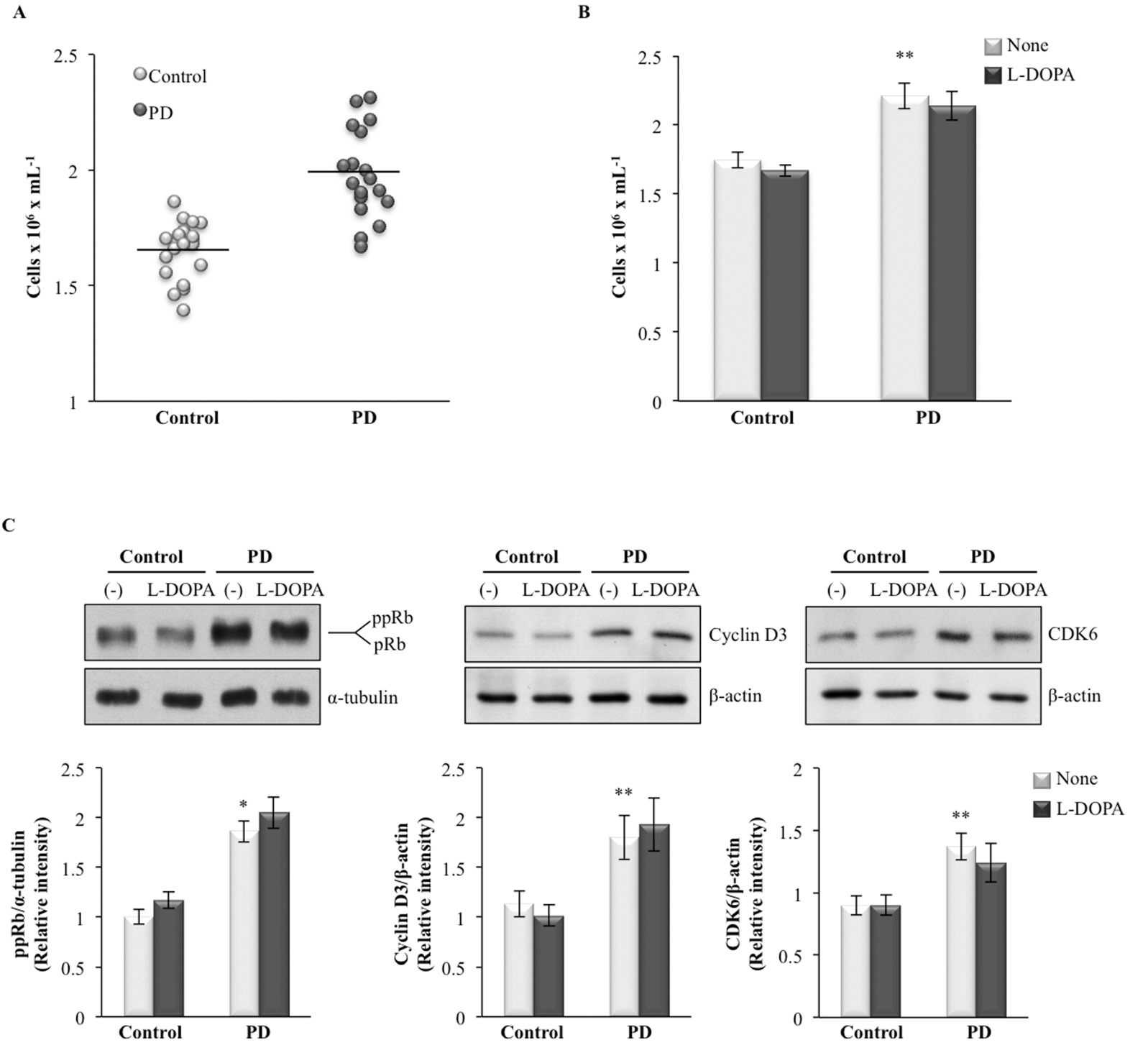
A
Control
PD
Cells x 10⁶ x mL⁻¹
2.5
2
1.5
1
Control
PD
B
None
L-DOPA
**
Cells x 10⁶ x mL⁻¹
2.5
2
1.5
1
0.5
0
Control
PD
C
Control
PD
(-) L-DOPA (-) L-DOPA
ppRb
pRb
α-tubulin
Cyclin D3
β-actin
CDK6
β-actin
ppRb/α-tubulin (Relative intensity)
Cyclin D3/β-actin (Relative intensity)
CDK6/β-actin (Relative intensity)
*
**
**
None
L-DOPA
Control
PD

Figure 1

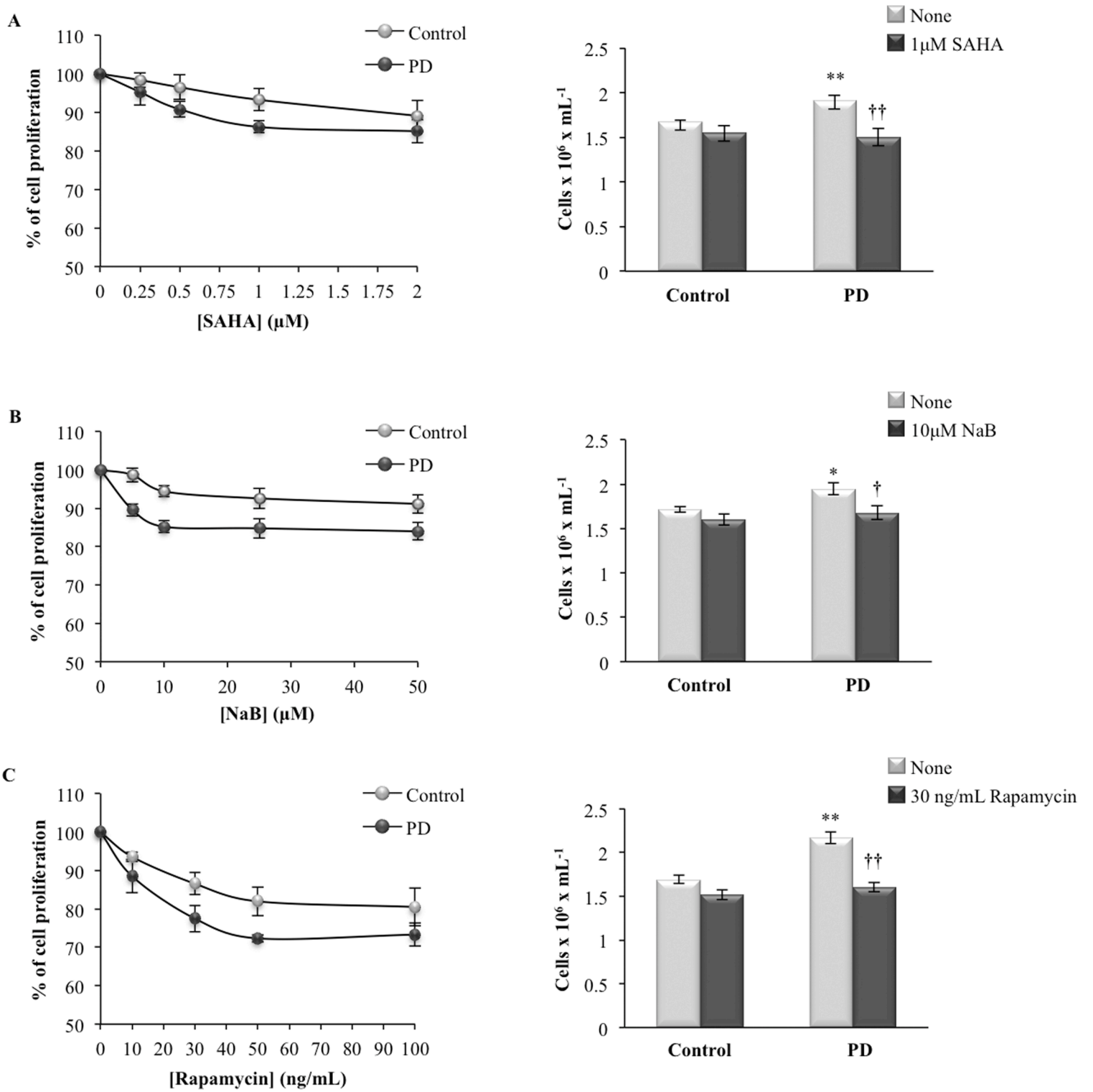
A
Control
PD
% of cell proliferation
[SAHA] (µM)
None
1µM SAHA
Cells x 10^6 x mL^-1
Control
PD
B
Control
PD
% of cell proliferation
[NaB] (µM)
None
10µM NaB
Cells x 10^6 x mL^-1
Control
PD
C
Control
PD
% of cell proliferation
[Rapamycin] (ng/mL)
None
30 ng/mL Rapamycin
Cells x 10^6 x mL^-1
Control
PD

Figure 2

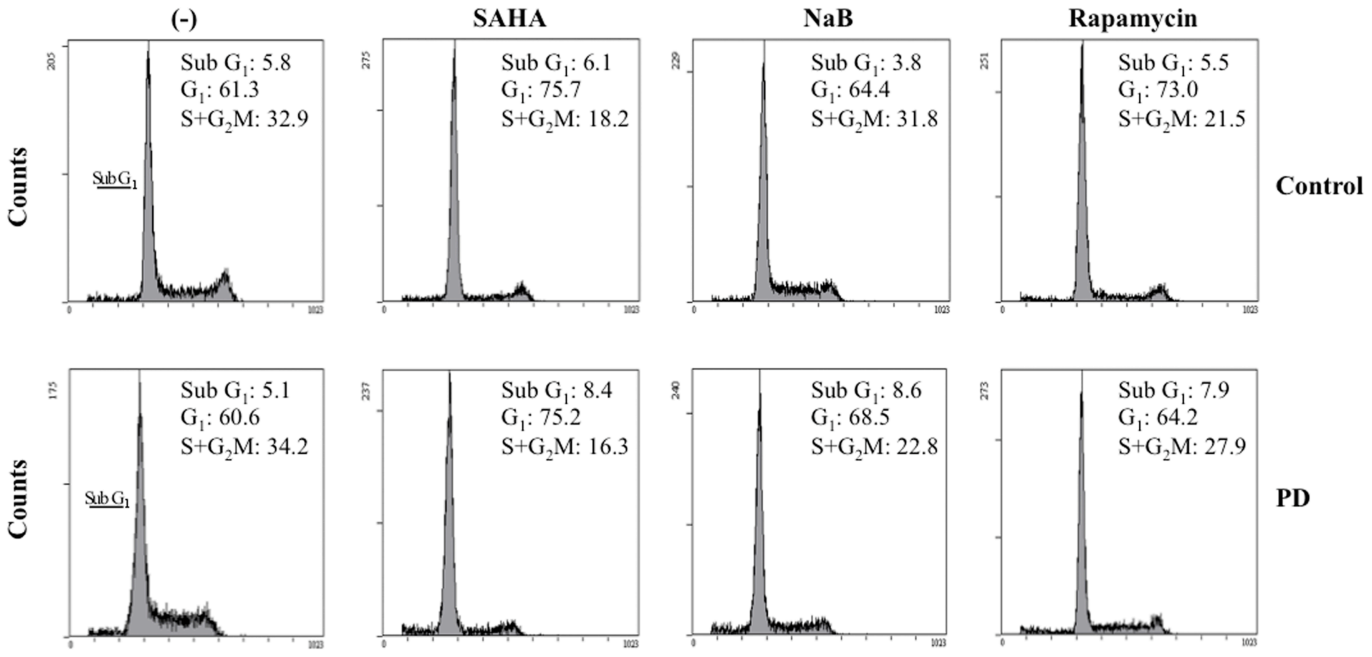
(-)
SAHA
NaB
Rapamycin
Counts
Sub G1: 5.8
G1: 61.3
S+G2M: 32.9
Sub G1
Sub G1: 6.1
G1: 75.7
S+G2M: 18.2
Sub G1: 3.8
G1: 64.4
S+G2M: 31.8
Sub G1: 5.5
G1: 73.0
S+G2M: 21.5
Control
Counts
Sub G1: 5.1
G1: 60.6
S+G2M: 34.2
Sub G1
Sub G1: 8.4
G1: 75.2
S+G2M: 16.3
Sub G1: 8.6
G1: 68.5
S+G2M: 22.8
Sub G1: 7.9
G1: 64.2
S+G2M: 27.9
PD

Figure 3

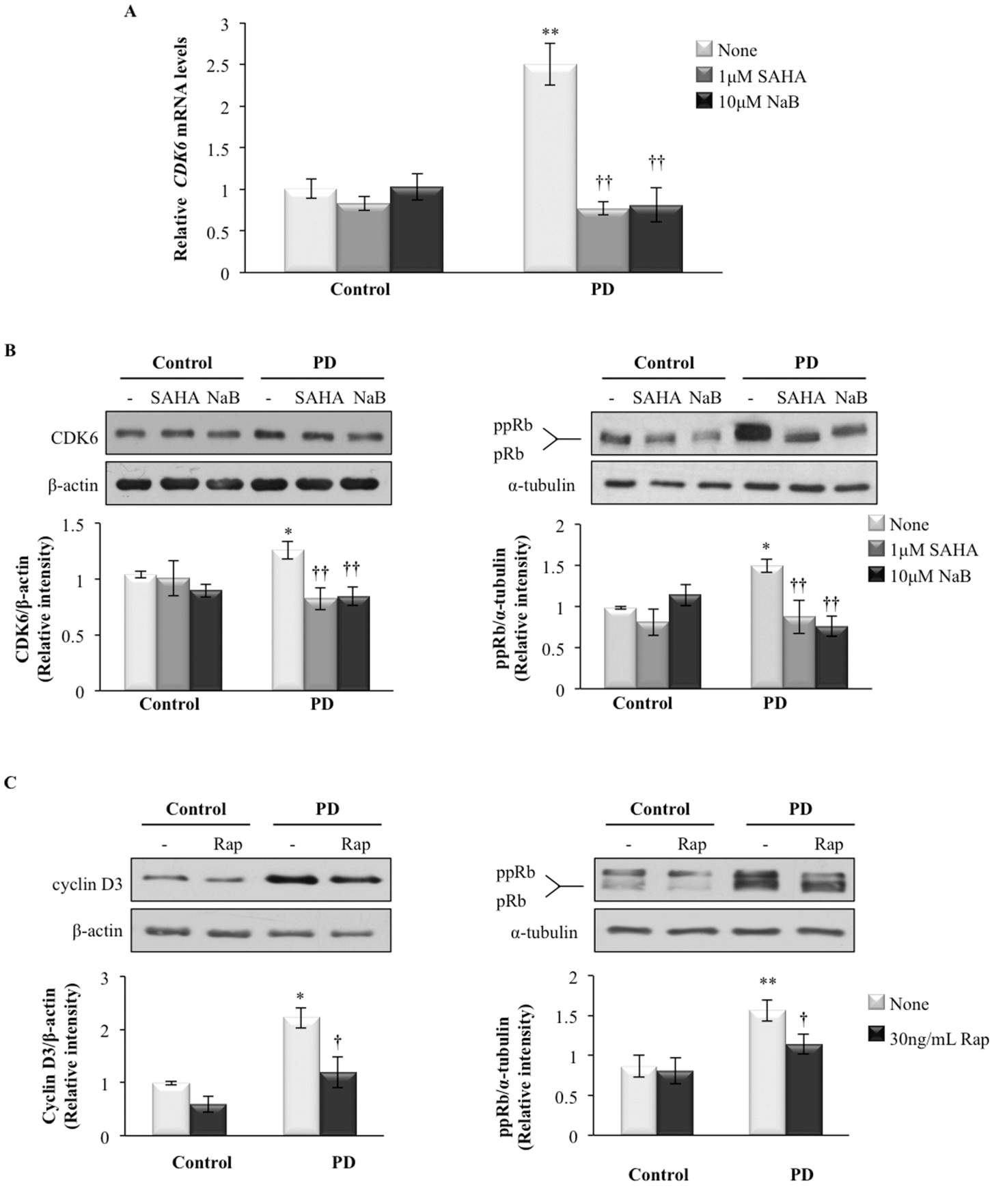
A
Relative CDK6 mRNA levels
3
2.5
2
1.5
1
0.5
0
**
††
††
None
1μM SAHA
10μM NaB
Control
PD
B
Control
PD
- SAHA NaB - SAHA NaB
CDK6
β-actin
CDK6/β-actin (Relative intensity)
1.5
1
0.5
0
*
††
††
Control
PD
ppRb
pRb
α-tubulin
ppRb/α-tubulin (Relative intensity)
2
1.5
1
0.5
0
*
††
††
None
1μM SAHA
10μM NaB
C
Control
PD
- Rap - Rap
cyclin D3
β-actin
Cyclin D3/β-actin (Relative intensity)
3
2
1
0
*
†
Control
PD
ppRb
pRb
α-tubulin
ppRb/α-tubulin (Relative intensity)
2
1.5
1
0.5
0
**
†
None
30ng/mL Rap

Figure 4

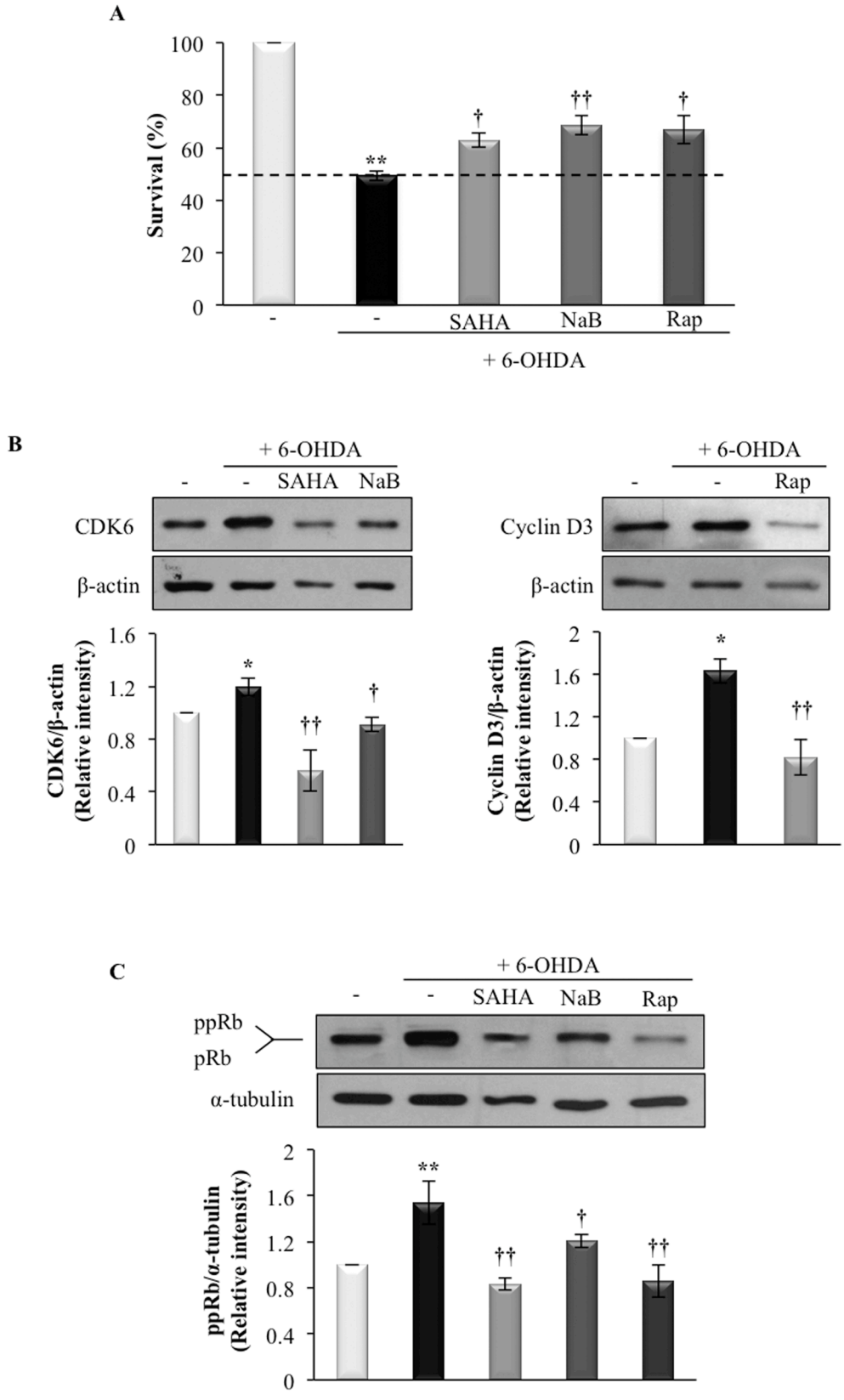
A
Survival (%)
100
80
60
40
20
0
**
†
††
†
-
-
SAHA
NaB
Rap
+ 6-OHDA
B
+ 6-OHDA
-
-
SAHA
NaB
CDK6
β-actin
CDK6/β-actin (Relative intensity)
1.6
1.2
0.8
0.4
0
*
††
†
+ 6-OHDA
-
-
Rap
Cyclin D3
β-actin
Cyclin D3/β-actin (Relative intensity)
2
1.6
1.2
0.8
0.4
0
*
††
C
+ 6-OHDA
-
-
SAHA
NaB
Rap
ppRb
pRb
α-tubulin
ppRb/α-tubulin (Relative intensity)
2
1.6
1.2
0.8
0.4
0
**
††
†
††

Figure 5

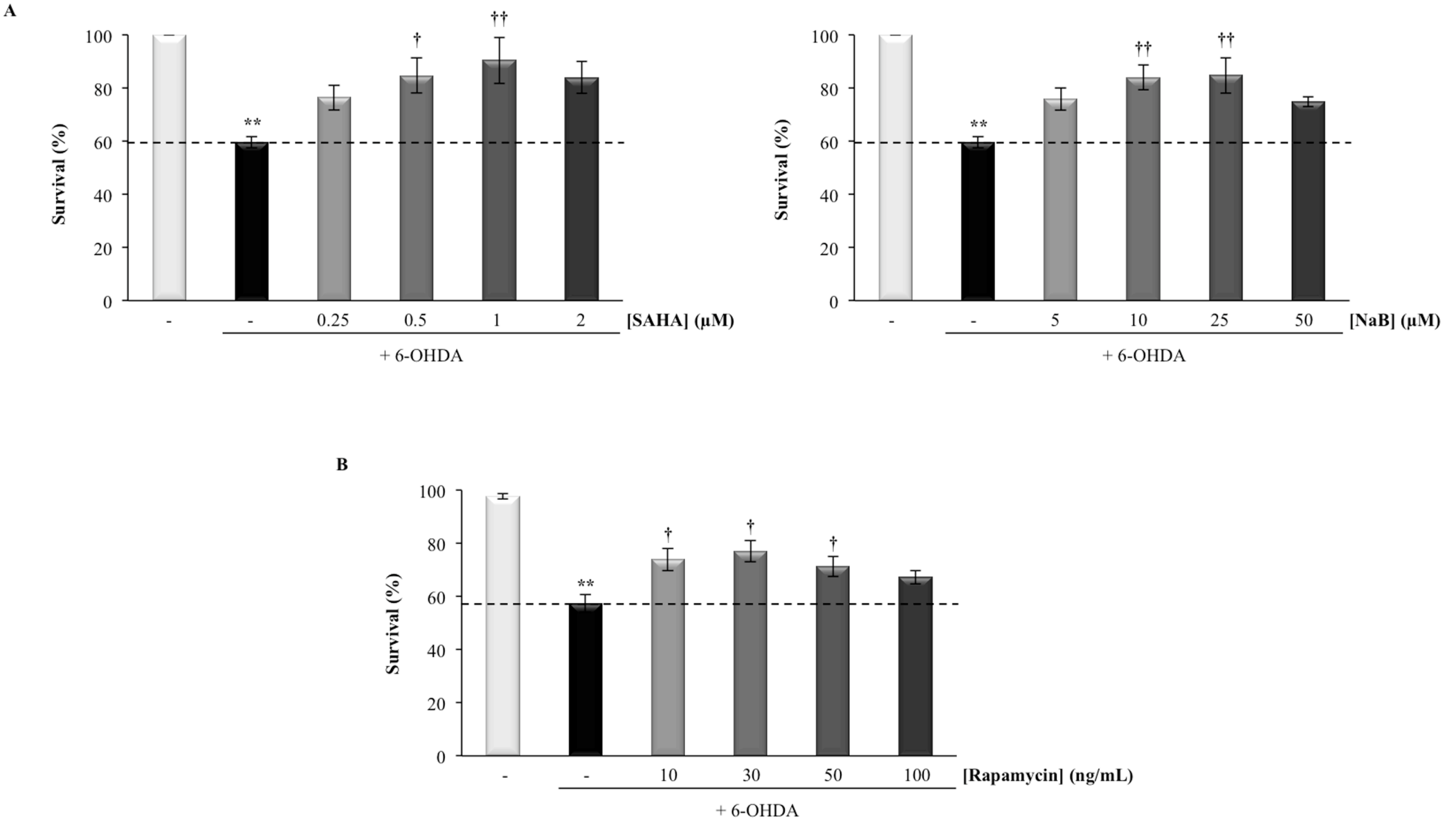
A
Survival (%)
100
80
60
40
20
0
**
†
††
-
-
0.25
0.5
1
2
[SAHA] (μM)
+ 6-OHDA
Survival (%)
**
††
††
-
-
5
10
25
50
[NaB] (μM)
+ 6-OHDA
B
Survival (%)
**
†
†
†
-
-
10
30
50
100
[Rapamycin] (ng/mL)
+ 6-OHDA

Figure 6

PARKINSON'S DISEASE

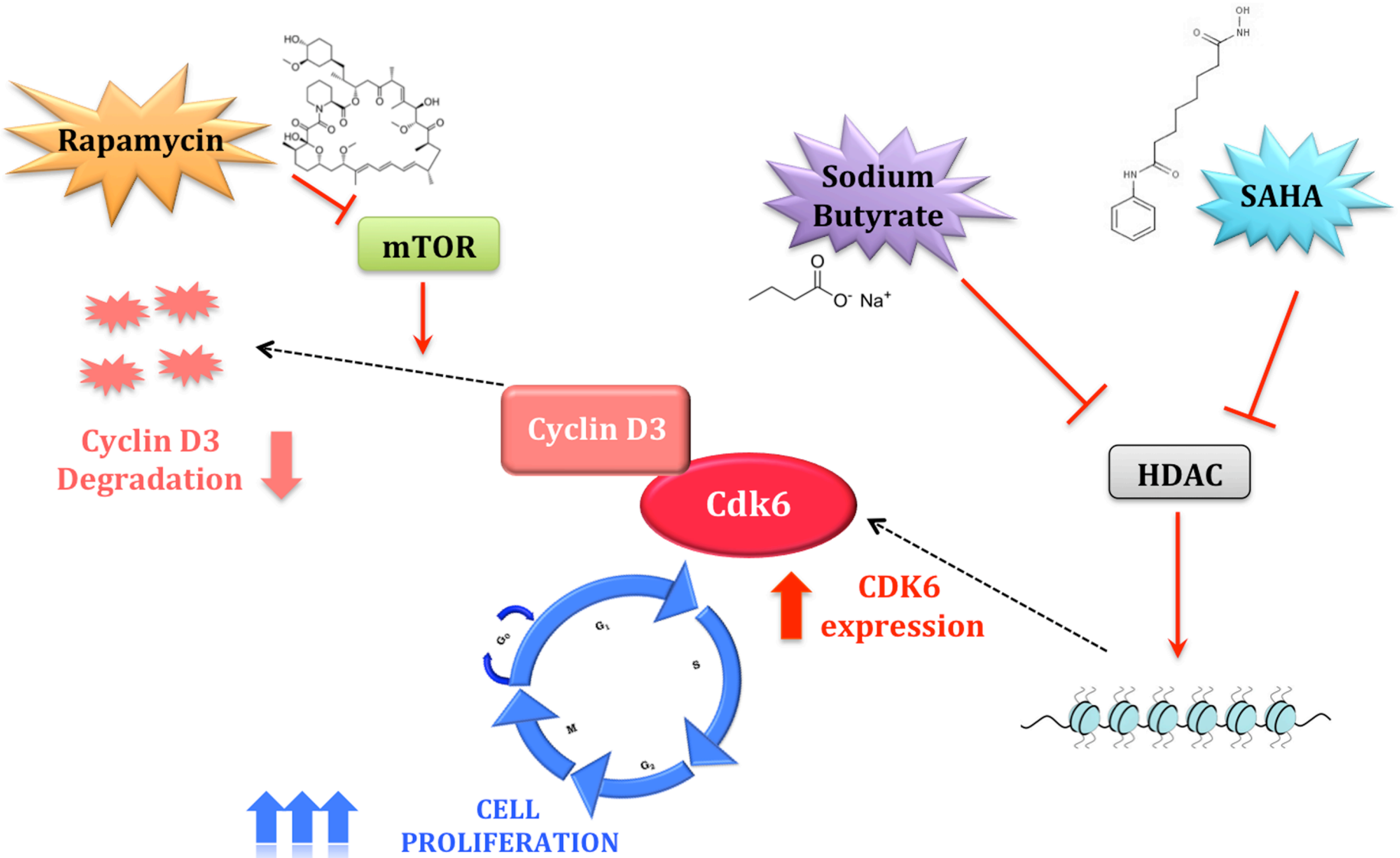

Figure 7