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Research Article
Effects of DNMT and HDAC Inhibitors (RG108 and Trichostatin A) on NGF-induced Neurite Outgrowth and Cellular Migration

Elif Kaya-Tilki, Miriş Dikmen and Yusuf Öztürk

Department of Pharmacology, Faculty of Pharmacy, Anadolu University, Tepebasi 26470, Eskişehir, Turkey

Abstract
Neuropathological characteristics of neurodegenerative disorders are atrophy or loss of specific neurons in the specific brain areas. Small-molecule products like epigenetic drugs, which can activate silenced genes, regulate chromatin remodelling and transcription, is presently been focused as an alternative approach for eliciting neuritogenic activity. Trichostatin A (TSA) has been effectively investigated for cancer treatment but it also has been shown to possess neuritogenic potential. In this study, it was aimed to compare the neuritogenic effects of two epigenetic drugs on PC-12 Adh cell line, which are the histone deacetylase inhibitor (HDACi) trichostatin A and a novel non-covalent DNA methyltransferase inhibitor (DNMTi) RG108. Cytotoxic effects were determined by MTT assay. Cell differentiation and migration analysis were evaluated by xCELLigence Real Time Cell Analyzer Dual Plate (RTCA DP) system according to the changing in Cell Index (CI) values. Cell migration analysis was also corroborated with morphological migration analysis. Matrix metalloproteinase-2 (MMP-2) levels related with neurite outgrowth were measured by ELISA. Finally, neurite outgrowth was observed with immunofluorescence staining and determined by neurite outgrowth analysis on PC-12 Adh cell line. The RG108 was found nearly as effective as TSA on neuronal differentiation, neurite outgrowth and cellular migration in PC-12 Adh cell line in a combination with nerve growth factor. According to the results, DNA methyltransferase inhibitors also hold promise in neurodegenerative disorders.

Key words: Neurodegeneration, neurite outgrowth, cell migration, epigenetics, trichostatin A, RG108

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Corresponding Author: Elif Kaya-Tilki, Department of Pharmacology, Faculty of Pharmacy, Anadolu University, Tepebasi 26470, Eskişehir, Turkey
Tel: +902223350580/3748

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The Central Nervous System (CNS) is characterized by a weak spontaneous regenerative capacity and has limited capacity of neurons to regrow injured axons (Oberbauera *et al*., 2013). The insufficiency of regeneration is based on a combination of different factors including reduced capacity of regeneration, lack of necessary trophic molecules to support growth and the presence of an environment hostile for any growth line (Woolf, 2003).

Neuroregeneration in the adult CNS is a multistep process that contains new neurons, generated through proliferation of endogenous stem/progenitor cells that will differentiate or the survival of injured neurons and both of them integrate into existing neural networks. Neurite outgrowth is the process in which neurons elongate their neurites to reach their targets (pathfinding), leading to the establishment of neuronal connections during development (Meur *et al*., 2009). The initiation and guidance of a neurite originate by several signal inputs from the extracellular space. The extracellular matrix (ECM) is a complex structure that contains chondroitin sulfate proteoglycans (CSPGs), which are produced by glial cells both during CNS development and injury (Kiryushko *et al*., 2004). The MMP-2 is one of the enzymes which is involved in the regulation of cell motility and neurite outgrowth via the degradation of inhibitory CSPGs for rendering the nerve more permissive to axon regeneration (Ferguson and Muir, 2000). Nerve Growth Factor (NGF) is also a extracellular matrix-associated neurotrophic factor (neurite promoter) that is essential for survival and differentiation of neuronal cells (Kiryushko *et al*., 2004; Huang and Reichardt, 2001; Segal, 2003). The NGF signaling is initiated when it binds to TrkA receptor at the cell surface, leading to transient activation of the Ras/MAPK pathway and phosphoinositide 3-kinase that promotes cell survival (Zhang *et al*., 2000). However, the delivery of exogenous neurotrophic factors is not effective enough for neurodegenerative diseases treatment. Neurotrophic factors are large polypeptide molecules that do not penetrate the blood brain barrier. In addition, they are metabolized by peptidases when administered peripherally (Maruoka *et al*., 2011). Therefore, potentiating the actions of NGF with small molecular weight compounds can be an alternative therapeutic approach.

Cellular and molecular changes can interact with genes and environmental factors to determine which cells age successfully and which go towards neurodegeneration (Hindle, 2010). Epigenetics refers to the reversible regulation of various genomic function that occur without a change in the DNA sequence and mediated through changes in DNA methylation and chromatin structure which can be an alternative approach in the neurodegenerative diseases treatment (Chouliaras *et al*., 2010).

DNA methylation and histone acetylation are interdependent and maintain equilibrium, allowing temporal expression of genes in normal cells, whereas in neurodegenerative disorders this balance might be disrupted. As HDACi, DNMTi are also low molecular weight compounds which are currently being developed for cancer therapy. Besides of anticarcinogenic effects, neuritogenic effects of HDACi were shown earlier, however the effects of DNMTi on neurite outgrowth and neuronal migration of PC-12 Adh cells, which respond to NGF with a dramatic change in phenotype and acquire a number of properties characteristic of sympathetic neurons (Greene and Tischler, 1976) are still not clear. In order to investigate the neuritogenic potential of DNMTi, the effects of RG108, a novel non-covalent DNMT1 inhibitor with lower cytotoxic properties according to the other agents in the group, was compared with a HDACi trichostatin A (TSA) on PC-12 Adh neurite outgrowth and cell migration.

MATERIALS AND METHODS

Cell culture: The PC-12 Adh (CRL-1721.1™) cell line was obtained from American Type Culture Collection (ATCC, USA) and maintained undifferentiated in DMEM medium containing 10% horse serum, 5% fetal bovine serum and 1% penicillin/streptomycin at 37°C in a humidified incubator with 5% CO_2.

To induce differentiation, 1% fetal bovine serum and 1% penicillin/streptomycin containing DMEM differentiation medium was used. Neuronal Growth Factor (NGF) was used as a co-stimulator in 100 nM concentration (Bang *et al*., 2001; Parker *et al*., 2000). TSA and RG108 was dissolved in DMSO and diluted to working concentrations with fresh medium. The highest concentration of DMSO was used in 200 μM which was less than 0.01%. Control group was prepared with medium containing 0.1% DMSO. The growth curve of each cell line was assessed.

Cell viability assay: In order to obtain non-cytotoxic concentrations of the compounds, neuronal viability was determined by MTT assay as described previously (Hansen *et al*., 1989). In short, cells were grown in 96-well plates at a density of 3×10^3 cells per well and subjected to different RG108 and TSA (10 and 100 nM; 1, 10, 100, 200 μM) concentrations. After 24 or 48 h incubation, MTT solution was added to reach a final concentration of 0.5 mg mL^{-1}. The cells

were incubated for another 4 h. Then, current medium was removed and 100 µL of DMSO solution was added. The absorbance was measured at 540 nm using a Cytation 3 Cell Imaging Multi-Mode Reader (Bio-Tek). Cell survival rates were expressed as the percentage of the DMSO (0.1%) solvent control.

Cell differentiation assay with Real-Time Cell Analysis system (RTCA DP): To investigate the differentiation of PC-12 Adh cells and determine the changing over time, a real-time cell analysis was performed with xCELLigence DP instrument by using E-plates that contain micro-electrodes and measuring electrical impedance which indicated as Cell Index (CI) value (a dimensionless parameter to represent cell status). This method is a modification of cell proliferation assay based on a characteristic feature of PC-12 cells, they stop proliferating with NGF signaling (Mustafa *et al*., 2015, 2016; Dwane *et al*., 2013). Concentrations were used as 10 and 100 nM, which showed lower cytotoxic effects according to the results of the MTT assay, alone or in combination with 100 nM NGF. Background of the E-plates were measured in 100 µL differentiation medium in the Real-Time Cell Analyzer (RTCA DP) station. Afterwards, cells were seeded in a 2×10^4 density per well on to 96-well E-plates (Roche Applied Sciences, Indianapolis, IN) in 100 µM differentiation medium. Cells were incubated up to 24 h and the impedance of each well was monitored using the RTCA device (xCELLigence, ACEA Biosciences, San Diego, CA) at 1 h intervals. After incubation the instrument was paused, 100 µL of the current medium was removed, twice of the concentrations were added into the wells and diluted to the final concentrations in differentiation medium. Assays were performed in octet and cell-free and concentration-free (with 0.1% DMSO) controls were run in parallel. Cells were monitored for up to 7 days at 1 h intervals in order to determine the cell differentiation comparatively, according to the CI values of the instrument. Neuronal differentiation percentage graph was drawn according to these CI values with GraphPad Prism V6 (http://www.graphpad.com/scientific-software/prism/).

Cell migration assay with Real-Time Cell Analysis system (RTCA DP): The RTCA DP instrument uses the CIM-Plate 16 for migration analysis, which contains microelectronic sensors connected on to the underside of the plate (Limame *et al*., 2012). For migration assay, the wells of the bottom chamber were filled with 160 µL of 10% serum containing DMEM medium and the top and bottom parts of the CIM-16 plates were assembled together. After the addition of 20 µL serum-free DMEM medium to the top chamber wells, the assembled CIM-plate 16 was allowed to stabilize for 1 h at 37°C, 5% CO_2. After the incubation, cells were seeded at a 2×10^4 density per well on to the top chambers of CIM-16 plates in 80 µL of serum-free media, then 100 µL of 100 nM NGF alone or combined with 100 nM RG108 and TSA concentrations were added. CIM-plate 16 was placed into the RTCA DP for data collection after 30 min incubation at room temperature. The RTCA DP software was set to monitor CI values once every 10 min for 72 h.

Morphological analysis of cell migration: Morphological migration analysis was performed with The Oris™ Cell Migration Assay kit (Platypus Technologies, LLC., Madison, WI) following the manufacturer's instructions. Briefly, cells were collected in differentiation medium at 5×10^4 cells mL^{-1} and 100 µL of the cell suspension was added to each well of the Oris plate. After 6 h incubation at 37°C in a 5% CO_2 incubator, the stoppers and then current medium were removed from the Oris plate. About 100 µL of 100 nM NGF alone or combined with 100 nM RG108 and TSA concentrations were added to wells. Attached cells were incubated for 72 h and every 24 h 3 wells from all concentrations were stained with 10 µL Hoechst 33258 for 5 min and photographed with an Oris detection mask in Cytation 3 Cell Imaging Multi-Mode Reader (Bio-Tek) using the blue filter cube.

Neurite outgrowth analysis: PC-12 Adh cells were plated on to the collagen IV coated 96-well culture plate (BD BioCoat™ Collagen IV Cellware) at a density of 2×10^3 cells per well in differentiation medium. Six hours after plating, medium was replaced with 100 µL of 100 nM NGF alone or combined with 10 and 100 nM RG108 and TSA concentrations. Seven days incubation period was determined according to the results of the differentiation analysis in RTCA DP system. On 7th day, morphometric analysis was performed on digitized images of live cells taken under a Leica DM 300 inverted microscope. Neurite outgrowth analysis was performed as described by Tamplenizza *et al*. (2013) with some modifications. Briefly, neurite growth was determined by manually tracing the length of the longest neurite and branch (if present) on images by using Leica LAS Image Analysis programme in pixels then converted to µm by using ImageJ (NIH). Totally 100 neurites were measured for each concentration group. Total length was divided to 100 to find the average neurite length per group. Experiments were repeated at least three times independently.

Quantifying MMP-2 levels: For quantifying the MMP-2 levels related with neurite outgrowth, an ELISA assay was performed.

Cells were seeded on a collagen IV coated 6-well plate at a 4×10^4 density per well in differentiation medium. After 6 h, medium was replaced with 100 nM NGF alone or combined with 10 and 100 nM RG108 and TSA concentrations containing 2 mL differentiation medium. On 7th day, supernatants of the cells were collected from wells for each group and stored at -80°C until the day of the assay was performed. A MMP 2 Rat ELISA Kit (USCN Life Science, Cat# SEA100Ra) was used and the analysis was performed according to the kit procedure.

Immunoflorescence staining: After neurite outgrowth analysis, cells were fixated with 80% methanol for 5 min. Cells were rinsed twice with 1×PBS then permeabilized with 0.1% PBS-Tween 20 for 20 min. After two washes in PBS, the cells were incubated in 1% BSA/5% fetal bovine 5% horse serum/0.3M glycine in 0.1% PBS-Tween solution for 1 h to permeabilise the cells and block non-specific protein-protein interactions. The anti-beta III tubulin antibody [2G10] (Abcam, Cat# ab78078, RRID: AB-2256751) was diluted 1:250 in the same solution and then the cells were incubated with 100 µL of antibody overnight at +4°C. After the incubation period, cells were rinsed twice with PBS and secondary antibody goat anti-mouse IgG H and L (Alexa Fluor®488), (Abcam, Cat# ab150113) at a 1:500 dilution was added to the wells and incubated for 1 h in room temperature. Hoechst 33258 (10 µg mL^{-1}) was used to stain the cell nuclei (blue) 10 min before the imaging with Cytation 3 Cell Imaging Multi-Mode Reader (Bio-Tek, USA).

Statistical analysis: Data were analyzed by one way ANOVA with Tukey's post-hoc, expressed as Mean±Standard error, p>0.05 not significant, p<0.05*, p<0.01** and p<0.001***.

RESULTS

Effects of trichostatin A and RG108 on cell viability: According to the MTT assay results, the cell viability significantly decreased with 200, 100 and 10 µM TSA concentrations for 24 and 48 h. On the other hand, 200 and 100 µM RG108 concentrations slightly decreased the cell viability (p>0.05 not significant) (Fig. 1). Non-cytotoxic concentrations were determined as 10 and 100 nM for further experiments.

Effects of trichostatin A and RG108 on cell differentiation: The CI values of concentrations without NGF and the control group obtained from RTCA DP instrument were like a plateau; however, the concentration groups containing NGF have showed a rapid increase after 4th day (Fig. 2a). Neuronal differentiation percentage graph was drawn according to the CI values on 4th day (Fig. 2b). According to the graphics, around 4th day, differentiation has been started and the experiment was terminated around 7th day because of the decrease in the CI values, that may be arising from the insufficient medium in the wells. The rapid increase in the CI values were accepted as the initial stages of differentiation (Dwane *et al*., 2013). Because during the initiation, cell body becomes larger which cause an increase in the impedance and then, the cell begins to extend neurites and cell body shrinks which cause a drop in the impedance.

Effects of trichostatin A and RG108 on cell migration: The most effective concentration on PC-12 Adh cell differentiation, 100 nM TSA and RG108 in a combination with 100 nM NGF, were choosen for migration analysis according to the results of the previous cell differentiation assay. The level of cell

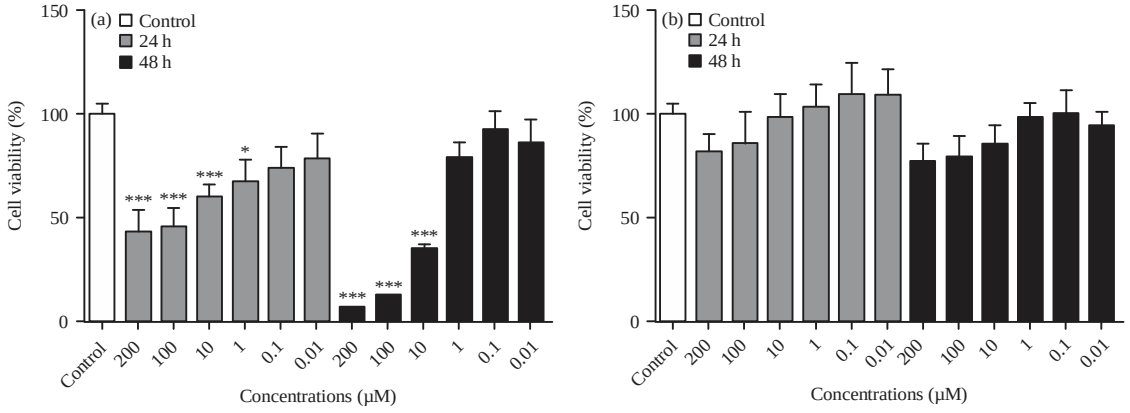

Fig. 1(a-b): Effects of (a) TSA and (b) RG108 on PC-12 Adh cell viability. The results are the means of 3 independent experiments. The error bars represent the standard deviations ((n = 8), p>0.05 not significant, *p<0.05 and ***p<0.001, compared to control)

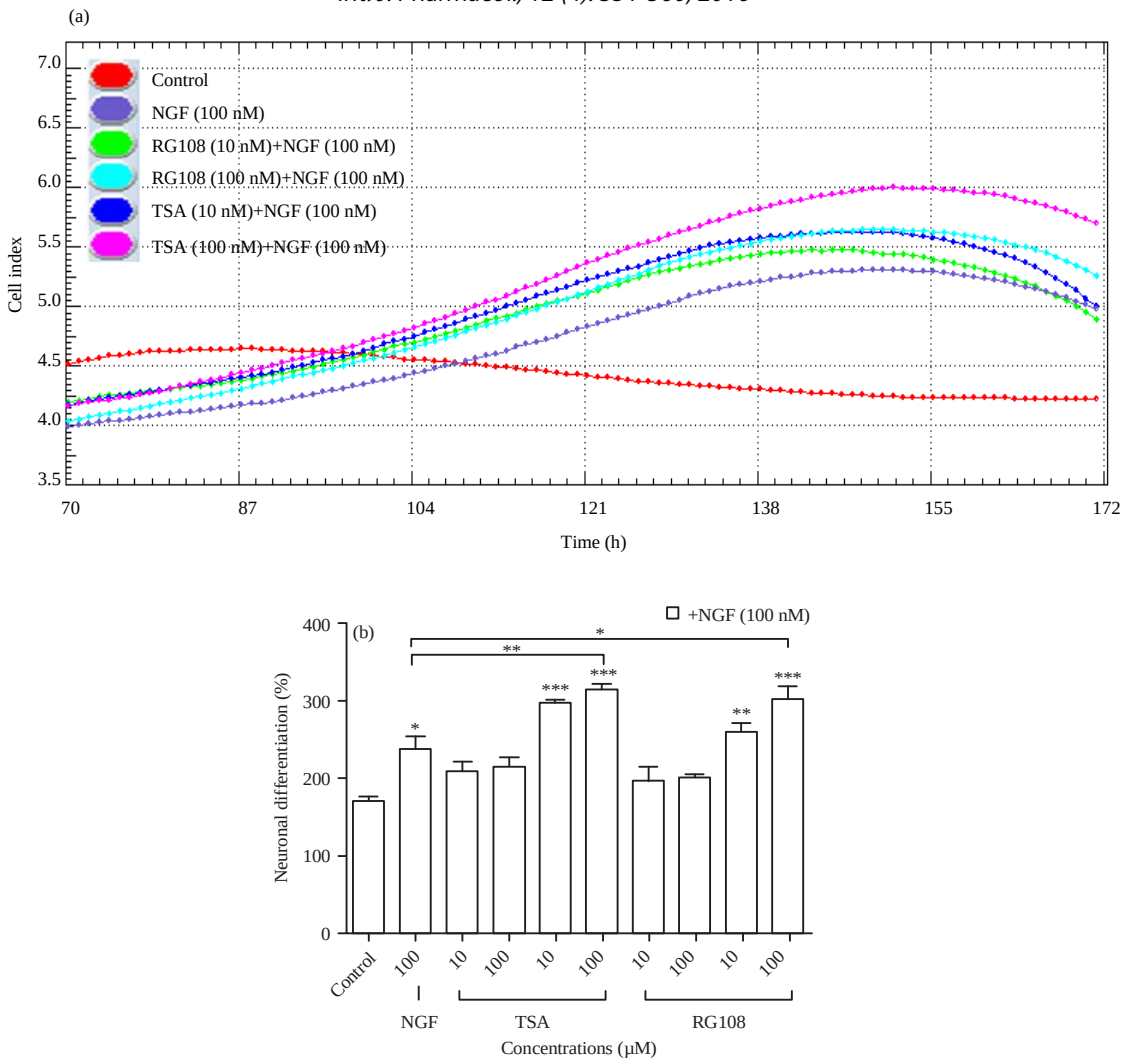

Fig. 2(a-b): Real-time monitoring of differentiative effects of (a) TSA and RG108 on PC-12 Adh cell line using RTCA-DP System (n = 8) and (b) Neuronal differentiation percentage graph was drawn according to these CI values p>0.05 not significant, *p<0.05, **p<0.01 and ***p<0.001, compared to control

migration was assessed by increases in the slope of the curve during active migration. Within the first 24 h, both TSA and RG108 combinations showed migration kinetics similar to that of NGF. In keeping with the results of the endpoint measurement, after 72 h, RG108 and TSA combination with NGF were caused higher migration compared with the positive control NGF (Fig. 3a). In addition to the results of migration analysis conducted with RTCA DP, according to the morphological migration analysis images shown in Fig. 3b, the effects of TSA combination was slightly higher than RG108 combination.

Effects of TSA and RG108 on NGF-induced neurite outgrowth: According to the results, 100 nM TSA in a combination with 100 nM NGF showed the highest neuritogenic effect (1430.7 µm) on PC-12 Adh cells. Average neurite length of the cells treated with 100 nM RG108 in a combination with 100 nM NGF was also showed slightly higher neuritogenic effect (1185.6 µm) than the cells treated with 10 nM combination groups. This data supports our neuronal differentiation and migration analysis results and taking together all this data may point a dose-dependent increase in neuritogenic effects (Fig. 4 and 5a).

Effects of TSA and RG108 on MMP-2 levels: After injury or degeneration, MMP-2 is involved in the regulation of neurite outgrowth via the processing of inhibitory CSPGs that are upregulated in the CNS and modulates cell migration through the release or inactivation of chemotactic signals (Rodriguez *et al*., 2010; Zhang *et al*., 2007). MMP-2 levels of

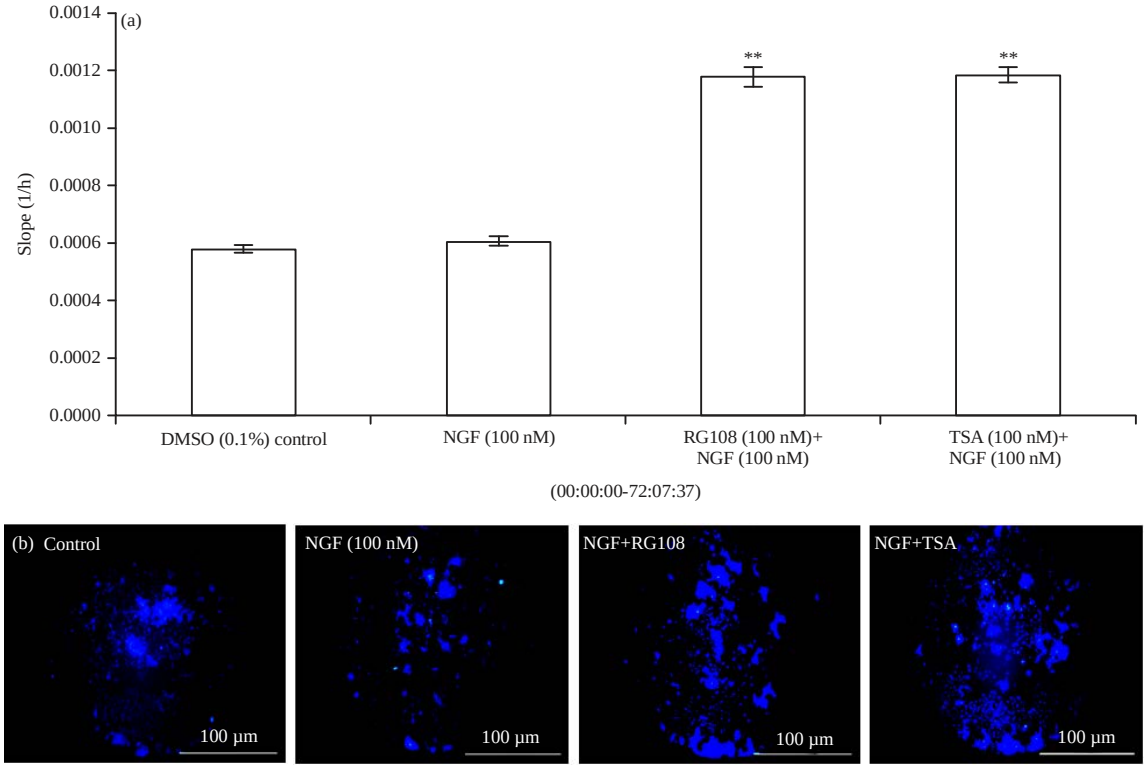

Fig. 3(a-b): Migration assay results were obtained with the xCELLigence RTCA DP system. (a) The slope of the migration curve was calculated using the RTCA 1.2.1 Software and (b) Morphological migration analysis images after 72 h, cells were stained with Hoechst 33258 and images were taken by Cytation 3 Cell Imaging Multi-Mode Reader with an Oris detection mask (n = 4), p>0.05 not significant and **p<0.01 and compared to control)

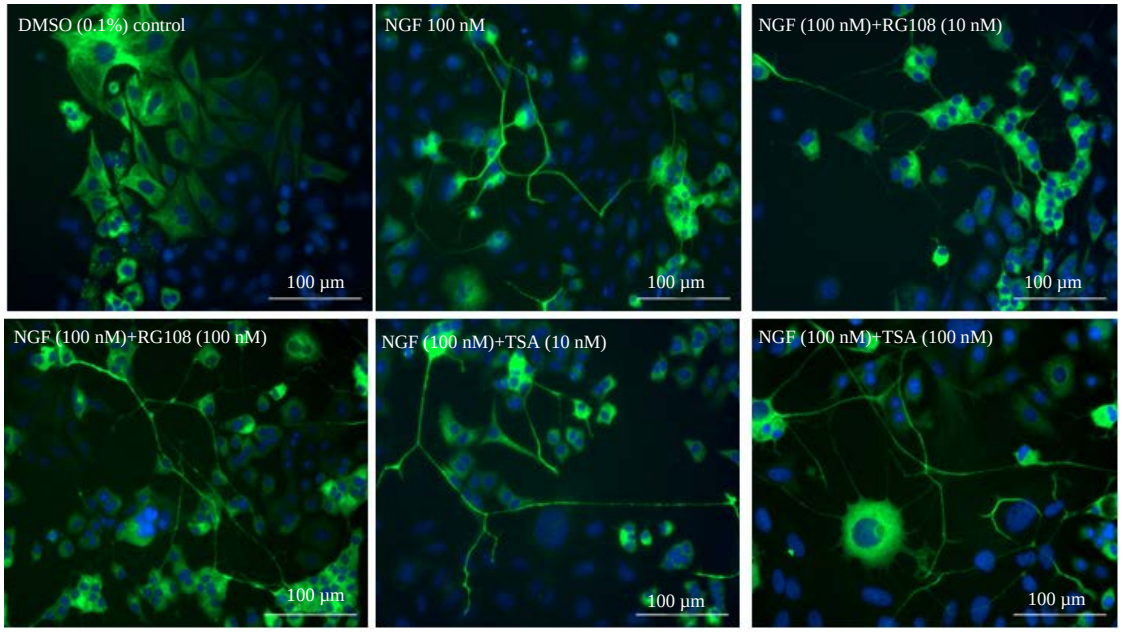

Fig. 4: Images of PC-12 Adh cells treated with various NGF, TSA and RG108 concentrations on 7th day. Cell nuclei were stained with Hoechst 33258 (blue) and tubulins were stained with AlexaFluor®488 (green). Images were taken with Cytation 3 Cell Imaging Multi-Mode Reader with 20X objective

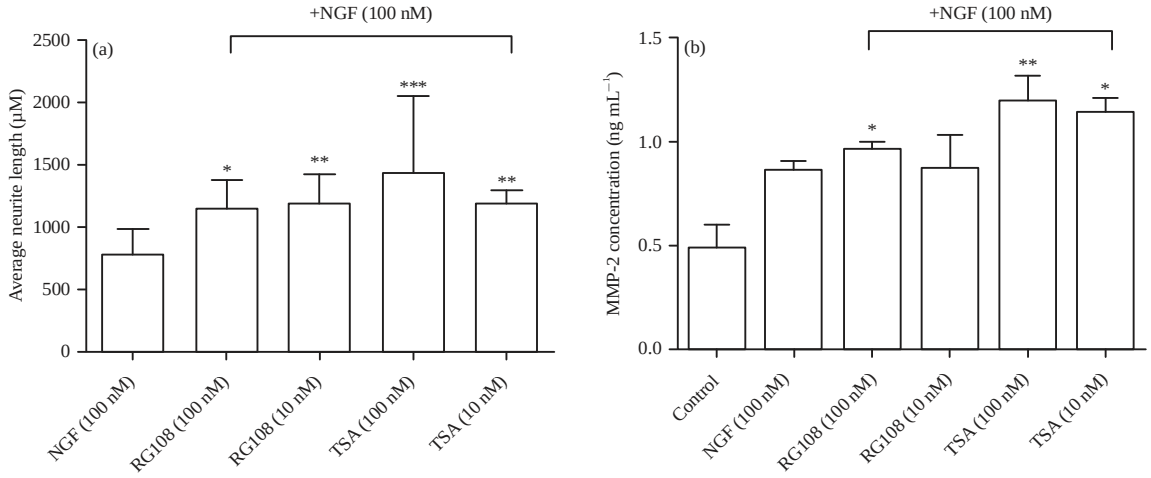

Fig. 5(a-b): Comparison of (a) Average neurite lengths and (b) Changes in MMP-2 levels. Data are Mean±SD values, p>0.05 not significant, *p<0.05, **p<0.01 and ***p<0.001

control, NGF (100 nM), NGF (100 nM) combination with TSA (100 and 10 nM) and RG108 (100 and 10 nM) were 0.48, 0.86, 0.96, 0.87, 1.20 and 1.14 ng mL^{-1}, respectively (Fig. 5b). According to the results, the MMP-2 levels of RG108 (100 nM) and TSA (10 and 100 nM)+NGF (100 nM) combinations were significantly increased compared with the control and NGF (100 nM) groups.

DISCUSSION

In this study, the epigenetic molecular mechanisms' relevance to neuronal differentiation were tried to understand. Histone deacetylase inhibitors (Sun *et al*., 2014; Cheng *et al*., 2012; Hamner *et al*., 2008; Choi *et al*., 2001) and DNMT inhibitors (Gnyszka *et al*., 2013; Savickiene *et al*., 2012a, b) are currently under development for cancer treatment. In terms of CNS applications, similar to the cancer therapy, where a disease treatment requires an extensive reorganization in epigenome, the drugs that can broadly affect the epigenome are desirable. Therefore, epigenetic drugs hold a great promise for the therapeutic efficacy in neurodegenerative disorders (Day *et al*., 2015). For this purpose, comparison of the effects of DNMTi RG108 and a HDACi TSA on PC-12 Adh neuronal differentiation and neurite outgrowth was done which is a very popular model for studying cell differentiation and have capacity to grow neurite-like processes in response to NGF (Greene and Tischler, 1976). Significant induction of neurite outgrowth and cellular migration with both RG108 and TSA synergistically with NGF were defined. This result was also supported with immunofluorescence imaging, morphologic and quantitative cell migration assay and measurement of MMP-2 levels related with neurite outgrowth which is an important process during neurite outgrowth. According to the results, RG108 is nearly as effective as TSA at 10 and 100 nM concentrations with lower cytotoxicity.

Neuronal injury and degeneration are responsible for various neurodegenerative disorders that are associated with loss of brain cells and axons resulting in functional deficits. Due to limited regeneration capacity of damaged neurons, the regulation of neurite outgrowth is crucial in developing strategies to promote axon and dendrite regeneration after nerve injury and in degenerative diseases (Wu *et al*., 2012). Cell migration establishes the formation of a highly specific pattern of connections between nerve cells and it is required for axonal guidance and neurite outgrowth and involves a number of coordinated signalling pathways (Dwane *et al*., 2013). Previous studies have indicated that neurite outgrowth and neuronal cell migration are important steps of neuroregeneration (Holtmaat *et al*., 1998; Struzyna *et al*., 2015).

Many different environmental factors can modulate epigenetic markers during development and in adult organism. In turn, epigenetics can cause and is associated with several neurodegenerative diseases. One potential method for preventing cell death and stimulating regeneration is to alter the intracellular signals which a cell uses to transduce responses to injury or growth-inhibiting substrates. For example, in apoptotic cell death, several transcription factors, including fas, p53, c-Jun, bax and bcl-2 are involved and may represent common pathways to intervene and prevent cell loss (Bredesen, 1995). Epigenetic modifications modulate gene expression, mediated by altering the transcription factor

activities, during several physiological processes, mainly during embryonic development and cellular differentiation (Nicolia *et al*., 2015).

Increased histone acetylation and transcription factor activity have been shown to protect from apoptosis in animal models of neurodegeneration (Saha and Pahan, 2006) and to promote neuronal differentiation (Balasubramaniyan *et al*., 2006; Schwechter *et al*., 2007; Maruoka *et al*., 2011). The TSA, directly inhibits histone deacetylases and allows histone acetyltransferases to hyperacetylate H3 at K9 and p53 at their C-terminus. The p53 forms transcriptional complexes with CBP/p300 and P/CAF. Then, in concert with CBP/p300 and P/CAF transcription co-factors, TSA causes to occupy specific promoters within the newly formed permissive chromatin state (Kiryushko *et al*., 2004; Tedeschi *et al*., 2009; Di Giovanni *et al*., 2006; Gaub *et al*., 2010). This situation results with the increased expression of neuronal outgrowth related genes like coronin 1b and GAP-43 (Kiryushko *et al*., 2004). Growth-associated proteins such as tubulin, actin and GAP-43 are thought to act as precursors of critical growth periods after injury and during development (Bisby and Tetzlaff, 1992; Hopker *et al*., 1999).

DNA methylation is also related with gene transcription which is mediated by DNMTs. In theory, a single cytosine methylation can completely silence the associated gene (Day *et al*., 2015). The RG108 is a novel DNMT1 enzyme inhibitor with low cytotoxicity and genotoxicity (Stresemann *et al*., 2006), this is the first paper that reports the neuritogenic effects of RG108 synergistically with NGF. The role of DNMT inhibitors on neurite outgrowth is still not clear but a study about the role of epigenetic reprogramming in cell differentiation which was investigated the NGF-induced differentiation of PC-12 cells was reported that the mRNA and protein levels of DNMT1 and DNMT3a decreased whereas, *de novo* methyltransferase DNMT3b increased during neurite outgrowth (Bai *et al*., 2005). Another study showed that upregulation of DNMT involved in motor neuron apoptosis and DNMT inhibitors, like RG108, protected motor neurons from apoptosis *in vivo* and *in vitro* (Chestnut *et al*., 2011). In addition, an interesting study was performed by Miller and Sweatt (2007) revealed that following contextual fear conditioning, DNMT gene expression is upregulated in the adult rat hippocampus and that DNMT inhibition blocks memory formation and also it activates the synaptic plasticity transcriptional gene reelin (Miller and Sweatt, 2007). Reelin is a highly expressed ECM glycoprotein gene during neuronal migration and neuroplasticity and responsible from cell to cell interactions (D'Arcangelo, 2005; Weeber *et al*., 2002). It regulates cell migration and positioning in the developing brain and also its function depends upon its proteolytic cleavage by MMPs and inhibition of MMPs causes a significant decrease in migration of neuron cells (Maurya *et al*., 2014; Tinnes *et al*., 2011; Ayoub *et al*., 2005). Therefore, according to the concentration groups in which higher MMP-2 levels were obtained, were also showed higher neuritogenic effects, this data might point a relation between reelin pathway.

CONCLUSION

In conclusion, promotion of an environment conducive to regrowth of the degenerated neurons by potentiating the effects of NGF with low molecular weight compounds, like RG108 and TSA, that can potantiate the action of neurotrophic factors might hold promise for counteracting neurodegeneration and with further investigations can be regarded as potential drug candidates. As another epigenetic modifier drug group, DNMTi also deserves interest in terms of these effects. Taking together all this data points that DNA methylation is a dynamic process which needs further investigations to understand in this field.

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