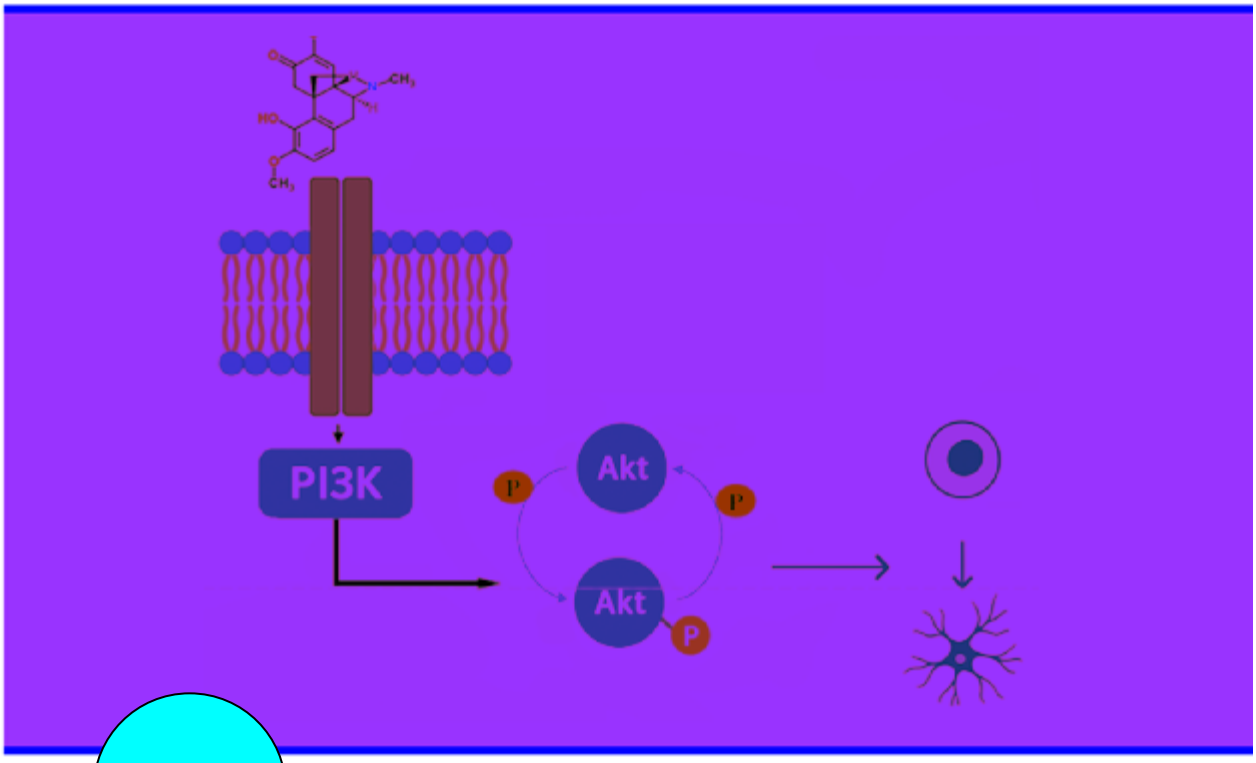

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Sinomenine promotes neuronal differentiation and neurite outgrowth via Akt activation *in vitro*

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Abstract

Sinomenine, a bioactive alkaloid from *Sinomenium acutum*, exhibits anti-inflammatory and neuroprotective effects, but its role in neuronal differentiation and morphological maturation remains unclear. This study used Neuro-2a cells and primary neural stem/progenitor cells (NSPCs) to examine the pro-differentiation effects of sinomenine. Sinomenine increased Neuro-2a cell viability at concentrations of 0.1–20 μM and showed no detectable cytotoxicity up to 100 μM. Under differentiation conditions, sinomenine enhanced neuronal differentiation and neurite outgrowth in Neuro-2a cells dose-dependently (5–50 μM), increased the proportion of β-tubulin III–positive neurons, promoted multi-neurite formation, and enhanced dendritic complexity in NSPCs. Mechanistically, sinomenine selectively induced Akt phosphorylation without activating ERK, p38, or JNK pathways, and pharmacological inhibition of Akt abolished sinomenine-induced neuritogenesis. These findings suggest sinomenine promotes neuronal differentiation and neurite outgrowth via the Akt pathway, supporting its potential in neuroregeneration.

Introduction

Neuronal differentiation and neurite outgrowth are fundamental processes underlying neural circuit formation and functional maturation of the central nervous system. These processes involve lineage commitment of neural stem/progenitor cells (NSPCs) followed by extensive cytoskeletal remodeling that supports axonal and dendritic extension (Llorente et al., 2022). Impairment of neurogenesis and neuritogenesis is a hallmark of neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease, for which current therapies remain largely symptomatic and ineffective at restoring neuronal loss (Wilson et al., 2023; Jiang et al., 2025). Consequently, identifying pharmacological agents capable of promoting endogenous neuroregeneration has become an important research focus.

NSPCs possess the capacity for self-renewal and multilineage differentiation, making them a promising cellular source for neuronal replacement and neural repair (Zhang et al., 2025). Enhancing neuronal differentiation

and morphological maturation of NSPCs through small molecules represents a feasible strategy for neuroregenerative intervention (Abdipranoto et al., 2008).

Neuro-2a is a mouse neural crest–derived cell line that is extensively used as an *in vitro* model to study neuronal differentiation, axonal growth, and associated signaling pathways. In recent years, natural compounds derived from medicinal plants have attracted attention as potential modulators of neuronal development and neuroprotection due to their structural diversity, multitarget activity, and favorable safety profiles. Several phytochemicals, including curcumin, resveratrol, and berberine, have been reported to promote neuronal differentiation and neurite outgrowth through activation of intracellular signaling pathways such as MAPK/ERK and PI3K/Akt (Liao et al., 2012; Ozpak and Bagca, 2024; Naveen et al., 2016; Jiang et al., 2023b).

Sinomenine is a natural morphinan-type alkaloid extracted from the roots and rhizomes of *Sinomenium acutum*, and has been widely used in traditional Chinese medicine for treating rheumatoid arthritis and chronic pain treatment (Lai et al., 2022) due to its potent anti-inflammatory (Jiang et al., 2023a; Wang et al., 2025; Zhang et al., 2019) and immunosuppressive properties (Zhang et al., 2021). Increasing evidence indicates that sinomenine exhibits neuroprotective effects in various neurological models by attenuating neuroinflammation (Rostami et al., 2022; Hong et al., 2022), oxidative stress (Zhang et al., 2019), and neuronal apoptosis (Ramazi et al., 2020; Jiang et al., 2020), and it is capable of penetrating the blood-brain barrier (Zhang et al., 2021). However, whether sinomenine directly regulates neuronal differentiation and neurite development remains largely unexplored.

Intracellular signaling pathways, particularly the PI3K/Akt pathway, play pivotal roles in regulating neuronal survival, proliferation, and neurite extension (Park et al., 2015), and their activation phosphorylates downstream targets involved in cytoskeletal dynamics and gene expression programs essential for neuronal maturation, such as GSK-3β, mTOR, and FoxO transcription factors (Wei et al., 2025; Vittori et al., 2021). Additionally, the MAPK/ERK pathway contributes to neuronal differentiation by promoting the expression of neurogenic transcription factors like NeuroD1 and Ngn1 (Liu et al., 2014), while p38-MAPK (Shinomiya et al., 2012) and JNK (St-Cyr et al., 2025) pathways often play context-dependent roles, either supporting or inhibiting neurite growth depending on stimulus duration and intensity; however, the precise signaling mechanisms mediating the effects of sinomenine on neuronal differentiation remain incompletely understood.

In this study, the effects of sinomenine on neuronal differentiation and neurite outgrowth were investigated in Neuro-2a cells and primary NSPCs, and the underlying signaling mechanisms were explored. The results suggest that sinomenine is a promising candidate for further development as a therapeutic agent for neurodegenerative diseases, where enhancing endogenous neuroregeneration is a major therapeutic goal.

Materials and Methods

Cell culture

Neuro-2a cells (CCL-131) were obtained from the American Type Culture Collection (USA) and cultured in MEM (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone) and 1 % penicillin/streptomycin (Beyotime, China). Cells were maintained at 37°C in a humidified incubator containing 5% CO_2.

Box 1: Differentiation and Neurite Outgrowth-Promoting Assay

Principle

The principle is to measure how neuronal precursor or model cells respond to neurogenic stimuli by extending neurites (axons and dendrites) and adopting differentiated neuronal characteristics

Requirements

24-Well plates; Antibodies (Beyotime, China); Dimethyl sulfoxide; ImageJ software; Fetal bovine serum (FBS); MEM medium; Neuro-2a cell lines; Phase contrast microscope (Olympus IX51, Japan); Penicillin/streptomycin; Phosphate buffer solution; Retinoic acid; Sinomenine.

Procedure

Neurite outgrowth assay

Step 1: Neuro-2a cells were seeded in 24-well plates at 1 x 10^4 cells/mL and cultured for 24 hours.

Step 2: The culture medium was then replaced with differentiation medium (MEM containing 0.5%FBS and 1%penicillin/streptomycin) and incubated for an additional 24 hours.

Step 3: Cells were subsequently treated with 0.1%dimethyl sulfoxide (DMSO, vehicle control), sinomenine (5, 10, 20, or 50 µM), or 10 µM retinoic acid (positive control) for 48 hours.

Inhibitors of signaling pathways

Step 1: Cells were pretreated for 1 hour with 10 µM specific inhibitors of Akt (MK2206, SF2712), ERK (FR180204, SD5978), p38 (BIRB796, SD5928), or JNK (SP600125, S1876, Beyotime) prior to incubation with 20 µM sinomenine for 48 hours.

Step 2: Neurite outgrowth was observed under a phase-contrast microscope, and neurites (defined as protrusions exceeding the diameter of the cell body) were quantified using ImageJ software.

Reference

Xiang et al., 2023

NSPCs were isolated from the brains of E13.5 ICR mice and maintained as previously reported (Xiang et al., 2017). For differentiation, single cells dissociated from neurospheres were seeded onto coverslips pre-coated with 100 ng/mL poly-L-ornithine (A-004-C, Sigma, USA) and 20 µg/mL laminin (CLS354232, Corning, Inc., USA) at a density of 2 × 10^4 cells/mL. The cells were then incubated in DMEM/F12 medium (Hyclone) supplemented with 10% FBS and 1% penicillin/streptomycin for 5 days to induce multilineage differentiation.

Cytotoxicity test

Cell viability was evaluated using the MTT assay (ST316, Beyotime, China) (Jiang et al., 2023b). Neuro-2a cells were seeded in 96-well plates at 5 × 10^3 cells/well and allowed to adhere for 24 hours, followed by treatment with sinomenine (0-100 µM; B20997, Yuanye, China) for 24 hours. After treatment, 100 µL of MTT solution (0.5 mg/mL in MEM) was added to each well and incubated for 4 hours. Formazan crystals were dissolved in 200 µL of DMSO, and absorbance was measured at 570 nm using a microplate reader. Cell viability was expressed as a percentage of the absorbance measured in the control group.

Immunostaining

NSPCs were treated with growth medium containing 0.1% DMSO served as a control or sinomenine (5, 10, 20, or 50 µM) for 5 days (Xiang et al., 2023). Cells were then fixed with freshly prepared 4% paraformaldehyde (P0099, Beyotime) for 20 min and permeabilized in PBS containing 0.4% Triton X-100. Following permeabilization, cells were blocked with PBS supplemented with 5% goat serum and 1% bovine serum albumin (BSA, P0007, Beyotime) for 20 min. Cells were subsequently incubated overnight at 4°C with anti-β-tubulin III antibody (T8660, Sigma) and then incubated with Alexa Fluor® 546-conjugated goat anti-rabbit IgG (1:2000, *v*/*v*, A-11010, Invitrogen, USA) for 1 hour at room temperature. Nuclei were counter-stained with 4',6-diamidino-2-phenylindole (DAPI, C1002, Beyotime), and images were acquired using a fluorescence microscope (Olympus IX71). Neurite complexity was analyzed by Sholl analysis using ImageJ software.

Western blot

Neuro-2a cells were seeded in 35 mm dishes at a density of 6 × 10^4 cells/dish and cultured to 70–80% confluence (Jiang et al. 2026). Cells were then incubated in differentiation medium for 2 hours, followed by treatment with 20 µM sinomenine for 0, 15, 30, 60, 120, or 240 min at 37°C. Cell lysates were prepared using ice-cold RIPA buffer (P0013B, Beyotime) supplemented with protease and phosphatase inhibitors (78443, Thermo Scientific, USA). After centrifugation at 14,000 x g for 15 min, protein concentrations were determined by a BCA Kit (P0010S, Beyotime), and 15 µg of protein per sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinyl-idene fluoride (PVDF) membranes (Millipore, USA). Membranes were blocked with 5%non-fat milk for 1 hour and incubated overnight at 4°C with primary antibodies against phospho-Akt1 (p-Akt, Ser473, AF1546), total Akt (AA326), phospho-extracellular signal-regulated kinases 1 (Thr202/Tyr204) /2 (Thr185/Tyr187) (p-ERK1/2, AF1891), total ERK1/2 (AF1051), phospho-p38 MAPK (Thr180/Tyr182) (p-p38, AM063), total p38 MAPK (AF1111), phospho-c-Jun N-terminal kinases 1/2/3 (Thr183/Thr183/Thr221) (p-JNK1/2/3, AF1762), total JNK1/2/3 (AF1048), and β-actin (AA128). The above antibodies were purchased from Beyotime. After incubation with HRP-conjugated secondary antibodies, immunoreactive bands were detected using enhanced chemiluminescence, and band intensities were quantified by densitometry (Tanon-5200, China).

Statistical analysis

All parameter values are expressed as the mean ± standard error of the mean (SEM). Data were subjected to one-way analysis of variance (ANOVA) and Tukey's test to assess the differences between the relevant control and each experimental group using GraphPad Prism 9 software (USA). A value of $p<0.05$ was considered statistically significant.

Results

Effect on Neuro-2a cell growth

The cytotoxicity of sinomenine on Neuro-2a cells showed no significant cytotoxicity was observed even at a concentration of 100 µM sinomenine (Figure 1). In contrast, at lower concentrations ranging from 0.1 to 20 µM, sinomenine significantly enhanced cell proliferation (ap<0.05, bp<0.01, cp<0.001 vs. the DMSO group).

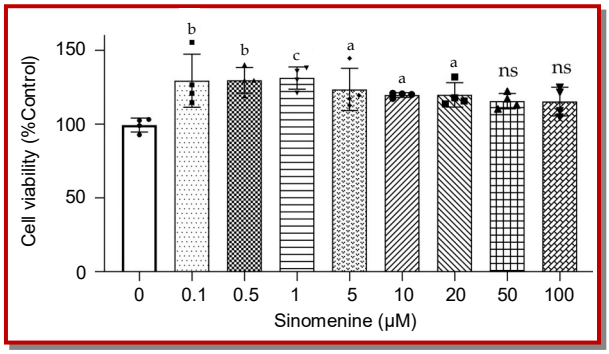

Figure 1: Cytotoxic effect of sinomenine on Neuro-2a cells was evaluated by MTT assay. Each column represents a quadruplicate experiment and data are presented as the mean ± SEM. Symbols indicate significant differences between sinomenine-treated groups and the DMSO control: [a]p<0.05, [b]p<0.01, [c]p<0.001, ns, not significant (p>0.05)

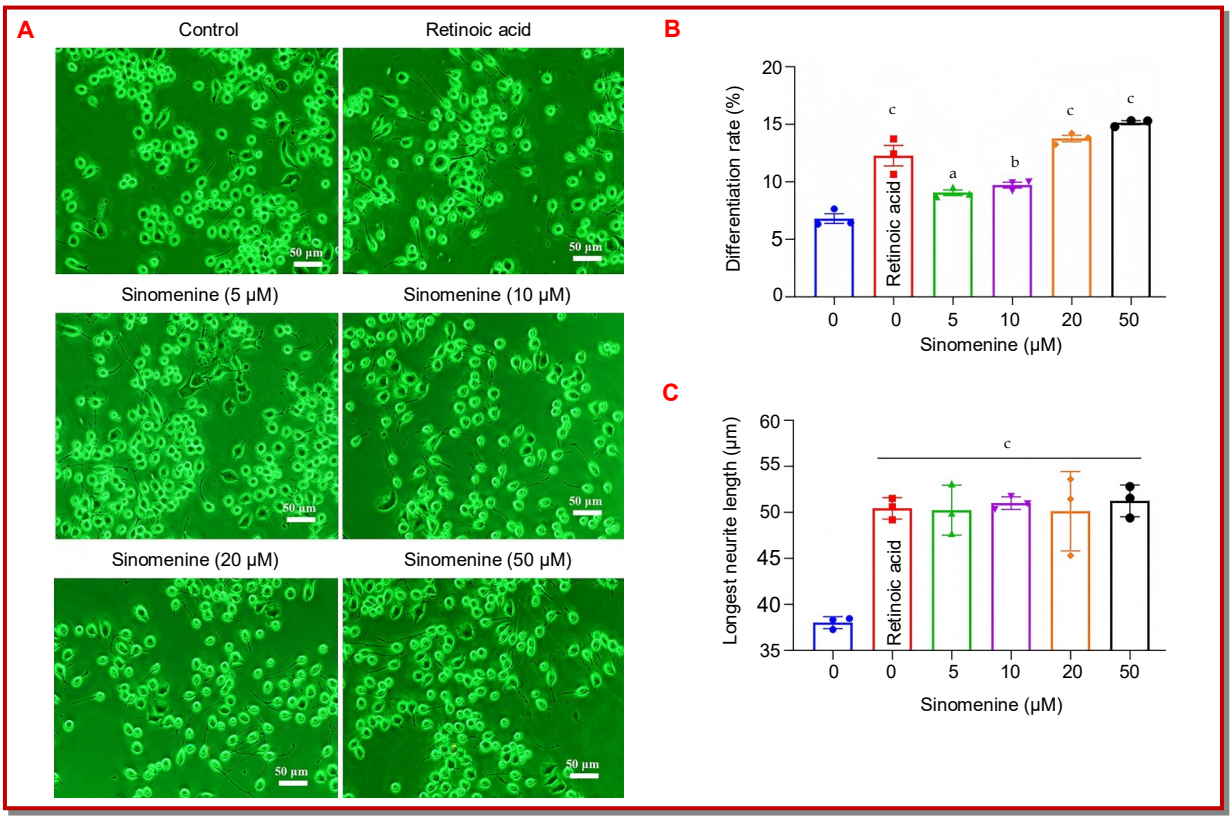

Figure 2: Effect of sinomenine on the differentiation and neurite outgrowth of Neuro-2a cells. The phase-contrast images of Neuro-2a cells treated for 48 hours with 0.1% DMSO (control), retinoic acid, or sinomenine at indicated concentrations (A). Quantification of the differentiation rate of the cells of Figure 2A, expressed as the percentage of cells with neurites longer than one cell body diameter (B). Measurement of the longest neurite length of each differentiated cell by Image J software (C). At least 500 cells/group were analyzed in each experiment. Data are shown as mean ± SEM (n = 3). $^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$ vs. DMSO group; one-way ANOVA followed by Tukey's test

Differentiation and neurite outgrowth in Neuro-2a cells

To explore the influence of sinomenine on the differentiation of Neuro-2a cells, the cells were cultured in differentiation medium with different concentrations of sinomenine and retinoic acid for 48 hours. Under the inverted phase-contrast microscope, the untreated cells (exposed to 0.1% DMSO) exhibited a predominantly round morphology with limited neurite outgrowth. In contrast, cells treated with retinoic acid and sinomenine displayed markedly elongated neurites (Figure 2A). Additionally, the percentage of differentiated Neuro-2a cells relative to the total cell population was calculated to quantitatively assess neuronal differentiation (Figure 2B), and the longest neurite length of each differentiated cell was measured and analyzed (Figure 2C). The results showed that sinomenine induced Neuro-2a cell differentiation in a concentration-dependent manner within the range 5 to 50 μM ($^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$ vs. the DMSO group), and sinomenine treat-ment could significantly increase the average length of the longest neurites in differentiated cells. Interestingly, 5 μM sinomenine was sufficient to induce neurite outgrowth to a similar extent as retinoic acid ($^{c}p<0.001$ vs. the DMSO group); however, increasing the concentration did not yield longer neurites. These results indicate that sinomenine exhibits potent activity in both differentiating Neuro-2a cells and stimulating neurite outgrowth.

Signaling pathway mediating sinomenine-induced neuronal differentiation in Neuro-2a cells

To understand which signaling pathways function in the sinomenine-induced neuronal differentiation and neurite outgrowth, Neuro-2a cells were treated with 20 μM sinomenine for different time points (0-240 min) and examined the levels of activated/phosphorylated forms of different molecules mediated by the potential signaling pathway (Figure 3). Notably, among the selected potential signaling molecules, only the expression of the phosphorylated Akt, p-Akt (S473), was markedly enhanced by sinomenine treatment (Figure 3A and 3B1, $^{c}p<0.001$ vs. 0 min). The phosphor-rylation levels of ERK1/2, p38, and JNK were not significantly affected following sinomenine treatment for up to 240 min (Figure 3A and 3B2-B4, ns or $p>0.05$ vs. 0 min).

Treatment of Neuro-2a cells with the specific inhibitors targeting key signaling molecules, followed by exposure to 20 μM sinomenine, revealed that MK-2206, a

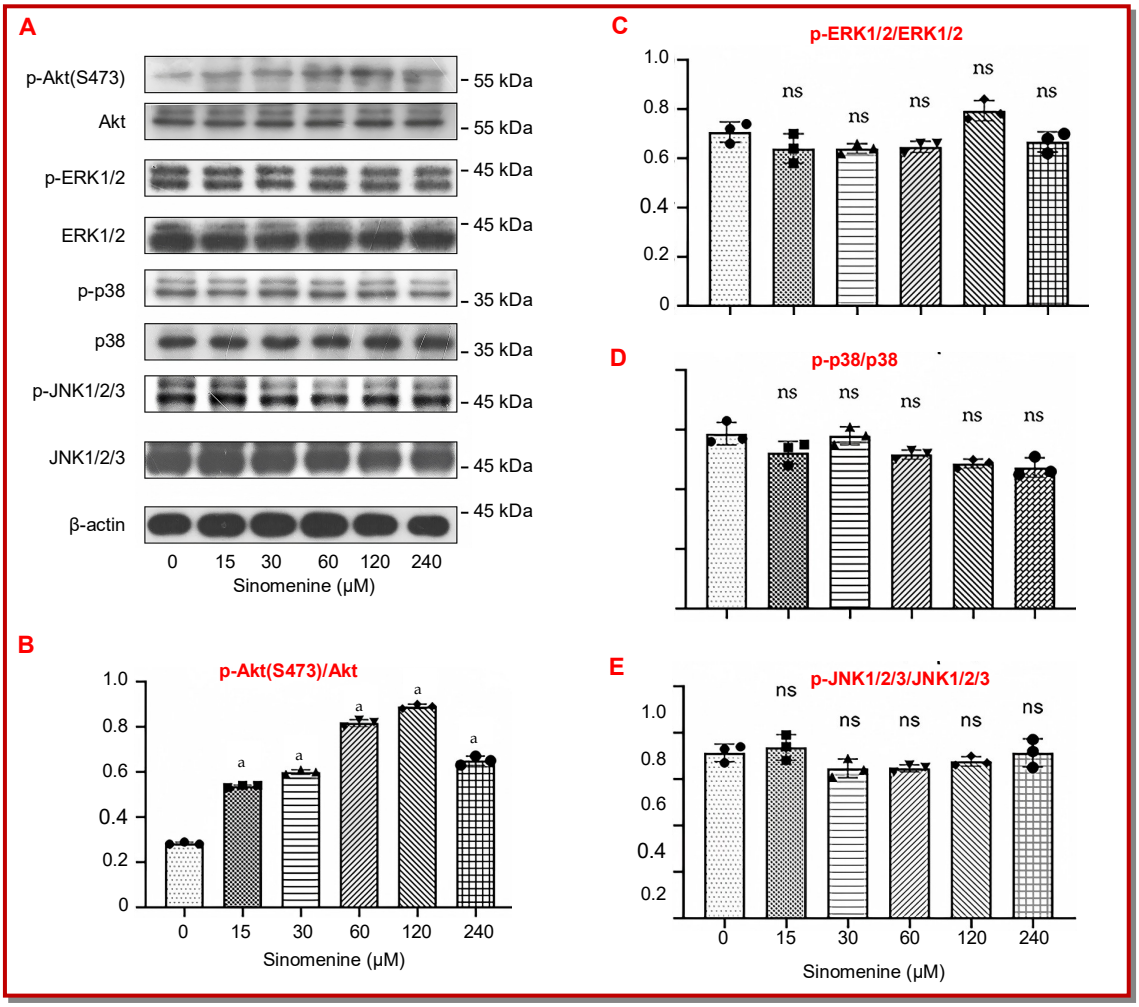

Figure 3: Effect of sinomenine on phosphorylation levels of Akt in Neuro-2a cells. Western blots showing the protein levels of phosphorylated and total Akt, ERK1/2, p38, and JNK in Neuro-2a cells treated with 20 μM sinomenine for the indicated durations. β-Actin was used as a loading control (A). Densitometric quantification of the phosphorylation levels normalized to the corresponding total proteins (B1-B4): p-Akt (Ser473)/Akt (B1), p-ERK1/2/ERK1/2 (B2), p-p38/p38 (B3), p-JNK/JNK (B4). Each column represents a triplicate experiment and data are presented as the mean ± SEM. [a]p<0.001 vs. 0 min; ns, not significant (p>0.05)

highly potent Akt inhibitor, significantly inhibited both the differentiation rate (Figure 4A and 4B, bp<0.01) and the longest neurite length (Figure 4A and 4C, bp<0.01) compared to sinomenine treatment alone. Moreover, the cell survival was reduced following Akt inactivation induced by MK-2206 (Figure 4A). Consistent with the western blot analysis that Akt were activated and promoted by sinomenine. By contrast, the promotive effects of sinomenine on differentiation and neurite outgrowth did not appear to involve ERK, p38, or JNK signaling, as pre-incubation with their respective inhibitors (FR180204, BIRB796, and SP600125) failed to attenuate the response (Figure 4A, 4B). Intriguingly, treatment with SP600125 appears to potentiate the stimulatory effect of sinomenine on the differentiation of Neuro-2a cells (Figure 4A and 4C, [b]p<0.01 vs. DMSO, [a]p<0.05 vs. sinomenine alone).

Effect on neuronal differentiation and morphological maturation as confirmed in NSPCs

The multipotent NSPCs, which readily give rise to neurons and astrocytes under specific conditions, were utilized to explore the influence of sinomenine on neuronal differentiation and neurite outgrowth in this study. As Figure 5A shows, abundant newborn differentiated neurons (β-tubulin III-positive or red-labeled cells) were observed in NSPCs after 5 days of treatment with different concentrations of sinomenine. The quantitative analysis (Figure 5B) found that the ratio of β-tubulin III positive cells was significantly increased from average 20.3% (DMSO) to 25.0% (5 μM sinomenine, [b]p<0.01), 30.0% (10 μM sinomenine, [c]p<0.001), 28.2% (20 μM sinomenine, [c]p <0.001), and 24.3% (50 μM sinomenine, [a]p<0.05), respectively. As illustrated in Figure 5A, neuronal maturation was accompanied by

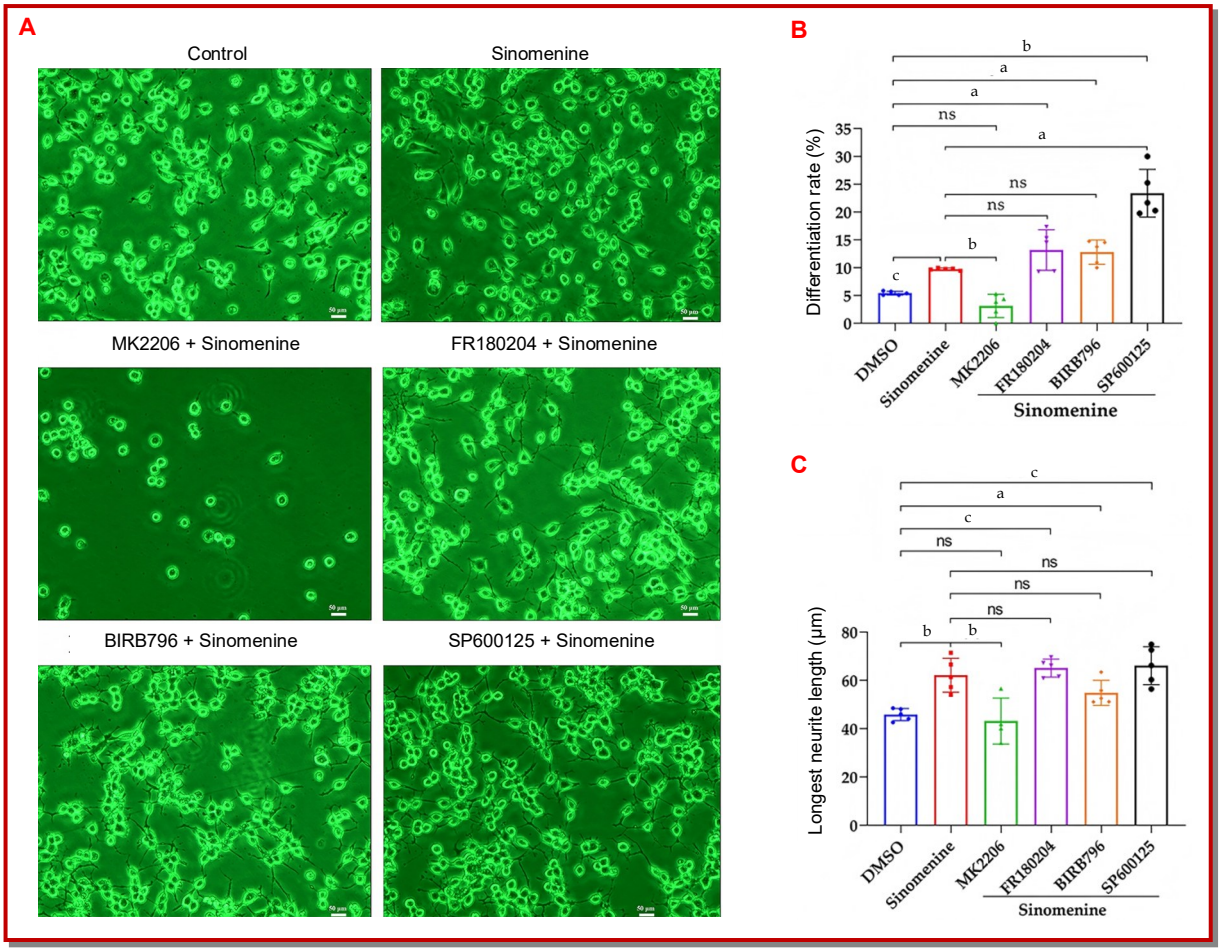

Figure 4: Activation of Akt signaling pathway for sinomenine induced neuronal differentiation and neurite outgrowth. The phase contrast images of Neuro-2a cells pre-treated for 1 hour with DMSO or 10 μM specific inhibitors targeting Akt (MK-2206), ERK (FR180204), p38 (BIRB796), or JNK (SP600125), followed by a 48-hours stimulation with 20 μM sinomenine. Scale bar: 50 μm (A). Quantification of neuronal differentiation rate (B) and the longest length of neurites per differentiated cell (C). Data are presented as the mean ± SEM from five independent experiments, with at least 400 cells analyzed per group. Symbols indicate significant differences between inhibitors + sinomenine-treated and sinomenine-alone group or DMSO control: $^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$, ns stand for $p>0.05$, i.e. not significant

pronounced morphological changes, including extensive neurite outgrowth, increased branching complexity, and a greater number of neurite branches per neuron. To gain insights into the effects of sinomenine during neuronal maturation, the total multi-neurite neurons (more than two branches) were measured for β -tubulin III-positive cells. With sinomenine treatment, the percentage of multi-neurite neurons were significantly increased from average 4.2% (DMSO) to 9.9% (5 μM sinomenine, $^{b}p<0.01$), 11.5% (10 μM sinomenine, $^{c}p<0.001$), 15.3% (20 μM sinomenine, $^{c}p<0.001$), and 21.1% (50 μM sinomenine, $^{c}p<0.001$), respectively (Figure 5C). Furthermore, the impact of sinomenine on dendritic complexity was evaluated using Sholl analysis (Figure 5D,E). The findings revealed that sinomenine facilitates the formation of more complex neurite structures in newborn neurons. These findings suggest that sinomenine enhances the neurite outgrowth and complexity of newborn neurons, ultimately promoting neuronal differentiation of NSPCs and fostering greater morphological maturity.

Discussion

The present study demonstrates that sinomenine directly enhances Neuro-2a cell proliferation at low concentrations (0.1–20 μM) without cytotoxicity under growth conditions. More importantly, sinomenine induces concentration-dependent neuronal differentiation and neurite elongation, with even 5 μM being sufficient to elicit an efficient differentiation response comparable to that of retinoic acid, a well-established inducer of neurite outgrowth (Janesick et al., 2015). Notably, although higher concentrations further increased the differentiation rate, they did not result in additional neurite elongation. This plateau effect suggests that sinomenine activates intracellular signaling pathways in a threshold

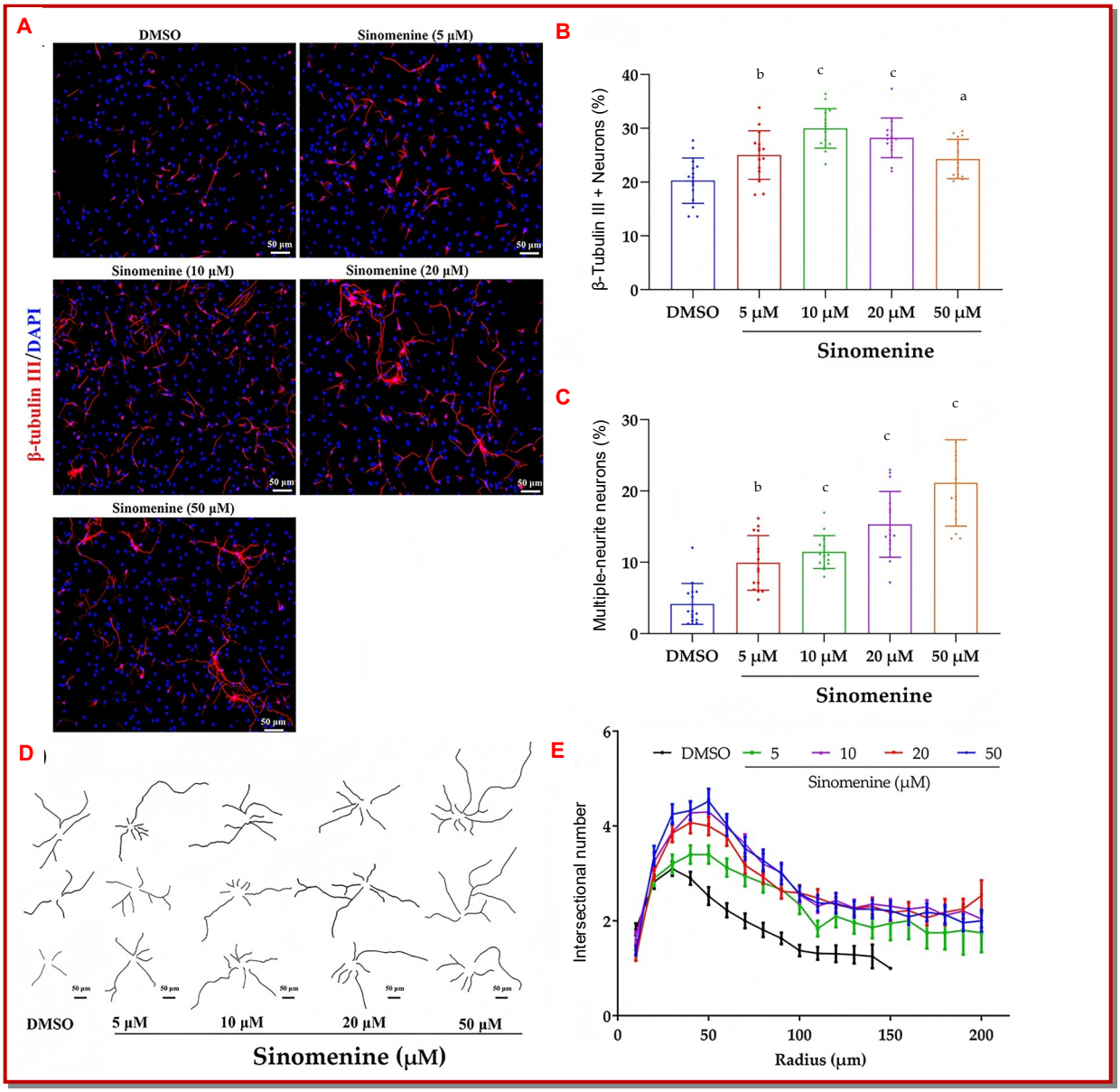

Figure 5: Effect of sinomenine on neuronal differentiation and morphological complexity in Neural Stem/Progenitor cells (NSPCs) towards a neuronal lineage. Immunofluorescence images of NSPCs after 5 days of treatment with sinomenine at indicated concentrations, note that newborn neurons were visualized by immunostaining for β-tubulin III (red), nuclei were stained with DAPI (blue). Scale bars, 50 μm (A). The percentage of β-tubulin III positive neurons (B) and proportion of multiple-neurite neurons (with more than two branches) among β-tubulin III positive cells were quantified (C). Tracings of neurite arborization for dendritic complexity analysis using Sholl analysis. Scale bar: 50 μm (D). Quantification of intersections per radius (E). Data are presented as mean ± SEM (n=40 neurons pooled from three independent experiments); [a]p<0.05, [b]p<0.01, [c]p<0.001 vs. DMSO group

-dependent rather than a linear dose–response manner.

Validation in NSPCs further confirmed that sinomenine not only increased the proportion of β-tubulin III–positive neurons but also markedly enhanced morphological complexity, as reflected by an elevated proportion of multi-neurite neurons and increased dendritic complexity revealed by Sholl analysis. These findings indicate that sinomenine not only initiates neuronal differentiation but also promotes subsequent morphological maturation, a process that is critical for the functional integration of newborn neurons into neural networks (Sultan et al., 2015).

Mechanistic investigations identified Akt phosphorylation as a key event in sinomenine-induced neuronal differentiation. Akt phosphorylation was markedly increased following sinomenine treatment, whereas pharmacological inhibition of Akt with MK-2206 abolished both neuronal differentiation and neurite outgrowth. These observations are consistent with the established role of the PI3K/Akt pathway in regulating

neuronal survival, cytoskeletal dynamics, and neuritogenesis (Sanchez-Alegria et al., 2018), and suggest that activation of survival signaling may underlie the proliferative effects observed at low sinomenine concentrations.

In contrast, inhibition of ERK, p38, and JNK signaling did not attenuate the differentiation-promoting effects of sinomenine, indicating that these pathways are not essential for its action. Interestingly, inhibition of JNK using SP600125 further potentiated the stimulatory effects of sinomenine. This enhancement may reflect off-target effects of SP600125 or may be attributable to previously reported roles of SP600125 in promoting neuronal growth and differentiation (Luo et al., 2022), enhancing neuronal survival (Guan et al., 2005), or relieving JNK-mediated suppression of neuronal differentiation in certain cellular contexts (Kanzawa et al., 2006).

A limitation of this study is that its direct molecular interactions with Akt and its downstream effectors, such as GSK-3β or mTOR, were not examined, and its efficacy *in vivo* warrants further validation.

Conclusion

This study identifies sinomenine as a natural compound that promotes neuronal differentiation, neurite outgrowth, and maturation via Akt signaling, supporting its potential for treating conditions associated with neuronal loss and impaired neurogenesis.

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Ethical Issue

The development, acquisition, authentication, cryopreservation, and transfer of cell lines between laboratories were followed according to the guidelines published in British Journal of Cancer, 2014

Conflict of Interest

Authors declare no conflict of interest

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