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Original article

pDok2, caspase 3 dependent glioma cell growth arrest by nitidine chloride

Ravindra Pramod Deshpande, Phanithi Prakash Babu*

Department of Biotechnology and Bioinformatics, School of Life Sciences, University of Hyderabad, Hyderabad, Telangana State, India

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ABSTRACT

Background: Nitidine chloride (NC) is known to exert anticancer and anti-metastatic effects on a variety of tumors. Recently, NC has also been shown to inhibit PIK3/AKT/mTOR axis in U87 human glioma cells.
Methods: The study shows NC employing pDok2, caspase 3 dependent cell death in C6 rat glioma and U87 human malignant glioblastoma cells. The effect of NC on glioblastoma cell lines was accessed by MTT, clonogenic and wound healing assays. Cell cycle analysis was performed by FACS. Moreover, the effect of NC on downstream target proteins, such as caspase3, pDok2, PARP, and Gsk3 beta, were measured by western blotting.
Results: Overexpressed pDok2 protein has recently been reported as a prognostic marker with poor outcomes for human glioblastoma multiformae. We found that NC inhibits pDok2 in U87 cells in a concentration-dependent way. We further showed that cleaved PARP and cleaved caspase 3 protein expressions were increased in C6 cells treated with NC in a dose-dependent way. NC effectively attenuated C6 cells growth and colony formation at 8 μM (micromoles) concentration. Cell cycle arrest in G2/M phase was further confirmed by flow cytometry. NC also exhibited its inhibitory effect on Gsk3 beta, which has been proven to be altered in glioma biology.
Conclusions: Collectively, we predicted that NC could be employed as a potential anti-glioma mediator that needs attention to explore the mechanisms of its activity.

Introduction

Gliomas are the cost common malignancies of the brain. In 2016, the World Health Organization (WHO) classification of central nervous system tumors has signified the use of molecular markers for diagnostic applications [1]. It has been reported that the Indian population succumbs to glioblastoma multiformae at a younger age than the Western population [2]. Glioma standard care treatment consists of near total/subtotal surgical resection/biopsy followed by radiotherapy and chemotherapy usually with temozolomide [3]. However, the present therapeutic regime fails to modify the average median age of patients' with glioblastoma multiformae to a significant extent [4]. At present, there is an urgent need to address this concern with novel therapeutic candidates to improve its prognosis and quality of life.

Nitidine is a phytochemical alkaloid derived from the roots of *Zanthoxylum nitidum*. Nitidine chloride (NC) is a chloride derivative of nitidine and possesses anti-inflammatory, analgesic, and antifungal bioactivities [5]. NC has been reported to inhibit hepatocellular carcinoma and renal cancer proliferation [6,7]. Studies on breast cancer showed that NC inhibits metastasis by suppressing c-SRC/focal adhesion kinase (FAK) pathway [8]. Recently, NC has been reported to inhibit malignant glioblastoma cells by targeting PIK3/AKT/mTOR axis [9].

The present study aimed at investigating the anti-glioma proprieties of NC on C6 and U87 glioma cell lines. The study established the migration and proliferation inhibitory effects of NC *in vitro*. It has also been shown that NC elevates caspase 3 and caspase 7 dependent cell death. Taken together, the present findings shed light on anti-glioma activity of NC and the potential need to understand its activity.

* Corresponding author.
E-mail addresses: ravi.deshpande3@gmail.com (R.P. Deshpande), prakash@uohyd.ac.in (P.P. Babu).

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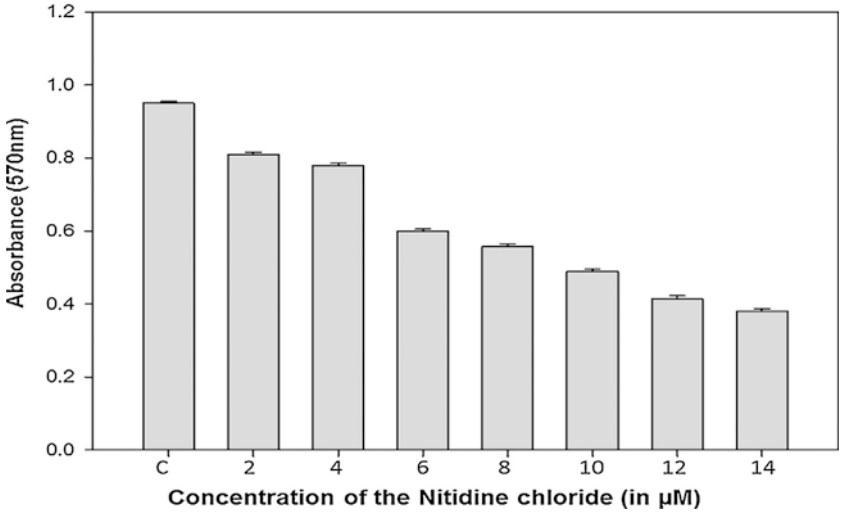

Fig. 1. Effect of nitidine chloride (NC) on U87 cell line (2–14 μM –24 h). NC was found to induce cell death in U87 cells in a concentration-dependent way. The degree of induced cell death at 8, 10, 12, and 14 μM was statistically significant as compared with control ($p < 0.05$).

Materials and methods

Cell lines and reagents

The C6 and U87 cell lines were procured from the National Center for Cell Sciences (NCCS) Pune, India and were cultured in DMEM medium (HIMEDIA, Cat no. AL007A-500ML) with 10% FBS (GIFCO, USA), antibiotic, antimycotic solution (GIFCO, USA, Cat no. 10270). Antibodies beta-actin (cat. No. 4970), cleaved caspase 3 (cat no. 9915), and Gsk3 beta (cat no.: 9315) were purchased from Cell Signaling Technology, USA. Secondary HRP antibodies and NC (cat. No. SML0610-5MG) were procured from Sigma-Aldrich, USA.

MTT assay for cell viability

C6 (1×10^4 cells/well) and U87 (1×10^4 cells/well) cells were seeded in 96 well plate and incubated at 37 °C at a concentration of 5% CO_2. After overnight incubation, cells were treated with different concentrations of NC (2–14 μM) for 24 h. After treatment, each well was added with 20 μl of 5 mg/ml of MTT dissolved in autoclaved miliQ and incubated for 3 h in dark. MTT was subsequently removed, and 50 μl of DMSO was added to each well. The plates were shaken at room temperature for 20 s, and the absorbance was measured at 570 nm by ELISA reader (Bio-Rad, USA). All experiments were repeated three times.

Colony formation assay

C6 cells were made in a single cell suspension. Around 500 cells/well were seeded in a six-well plate and incubated for 24 h at 37 °C with 5% CO_2. The cells were then treated with three different concentrations of NC (4, 8, and 12 μM) for 24 h. Media in the six-well plate was replaced with fresh one and cultured for 8 days. The media was again refreshed after 3 days. The cells were then fixed with 4% paraformaldehyde (PFA) for 10 min at room temperature. Further staining with 2% crystal violet was performed. Visible colonies were counted. Images were taken with Olympus digital camera (Olympus, Japan).

Cell cycle analysis by flow cytometry

Flow cytometry was used to analyze the distribution of cells upon NC treatment. C6 cells were seeded at 1×10^5 cells/well density in the six-well plate for 24 h. The cells were then treated with NC for 24 h. Total cells were collected by centrifugation

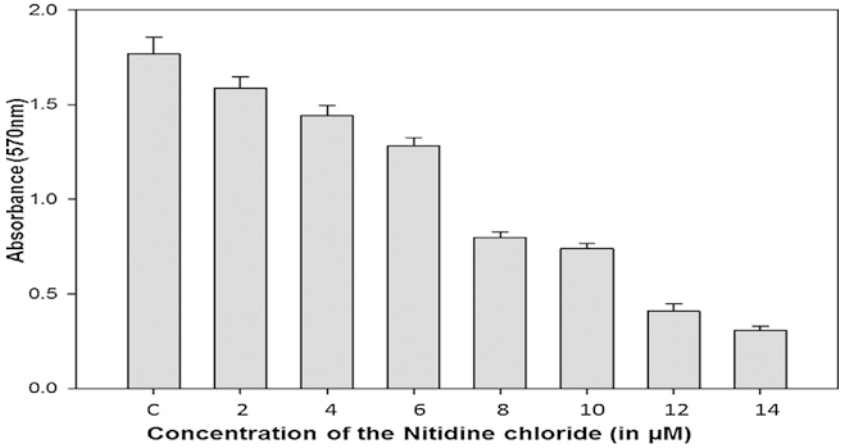

Fig. 2. Effect of nitidine chloride (NC) on C6 cell line (2–14 μM –24 h). NC was found to induce cell death in C6 cells in a concentration-dependent way. The degree of cell death induced at 8, 10, 12, and 14 μM was statistically significant as compared with control ($p < 0.05$).

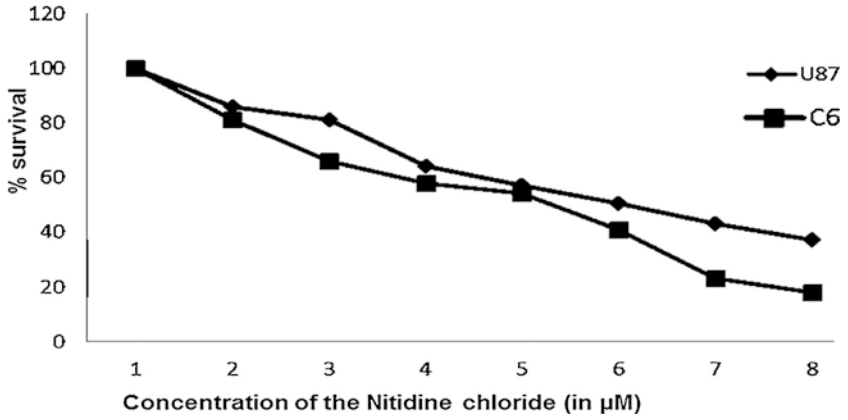

Fig. 3. Effect of nitidine chloride (NC) on U87 and C6 cell line (24 h). C6 and U87 cells were observed with IC50 value of around 8 µM.

(Eppendorf centrifuge- 5418R) at 1500 rpm for 5 min and washed with phosphate buffer saline (PBS). The cells were then fixed with 70% ethanol for 30 min at room temperature. Cells were collected by centrifugation at 2000 rpm for 5 min and washed with PBS. Further staining was done with propodeum iodide (50 µg/ml) and RNase A (1 mg/ml) solution. Cell cycle analysis was performed with BD LSRFortessa flow cytometer (BD Biosciences, USA). The data was analyzed using FlowJo (Oregon) analysis platform.

Wound healing assay

C6 cells (1×10^5 cells) were seeded in a six-well plate. After the cells reached confluency, a wound was marked with a blunt tip. Dislodged cells were removed, and fresh 2 ml medium supplemented with 10% serum was added. The cells were then treated with NC. Percentage inhibition of wound healing was assessed in treated experimental sets as compared to control.

Western blot

C6 wells were seeded in 20 mm Petri plate with 3 ml of DMEM media supplemented with 10% of serum. After the cells reached 70% of confluency, they were treated with NC for 24 h. Control cells were treated with vehicle control (DMSO). After 24 h, the cells were washed with PBS and lysed in RIPA buffer supplemented with protease and phosphate inhibitor cocktail (Sigma, cat no.: P8340). The cell lysate was then used for protein estimation by Bradford's reagent (Sigma, cat. No. B6916). An equal amount of protein (50 µg) was mixed with 6x gel loading dye. It was boiled for 3 min and used for loading with 12% SDS polyacrylamide gel. Proteins in the gel were transferred to nitrocellulose membrane (Millipore) with Towbin buffer overnight at 25 V for 12 h. Cleaved caspase 3, Gsk3 beta primary antibodies were dissolved in 5% skim milk powder mixed with TBST (1:500 dilution for each antibody). Blots were blocked with 5% skim milk solution for 1 h followed by primary antibody incubation overnight at 4 °C. After subsequent washing with TBS and TBST, the blots were probed with secondary antibody (1:10000 dilution) for 1 h at room temperature. The blots were then developed with VersaDoc (Bio-Rad, USA) molecular imager.

Statistical analysis

The results were expressed as mean +SD. SigmaPlot 11.0 was used for statistical analysis. Student's *t* test was used to analyze the other data. Error bars represented standard error of three independent experiments. Values of $p < 0.05$ was considered as statistically significant.

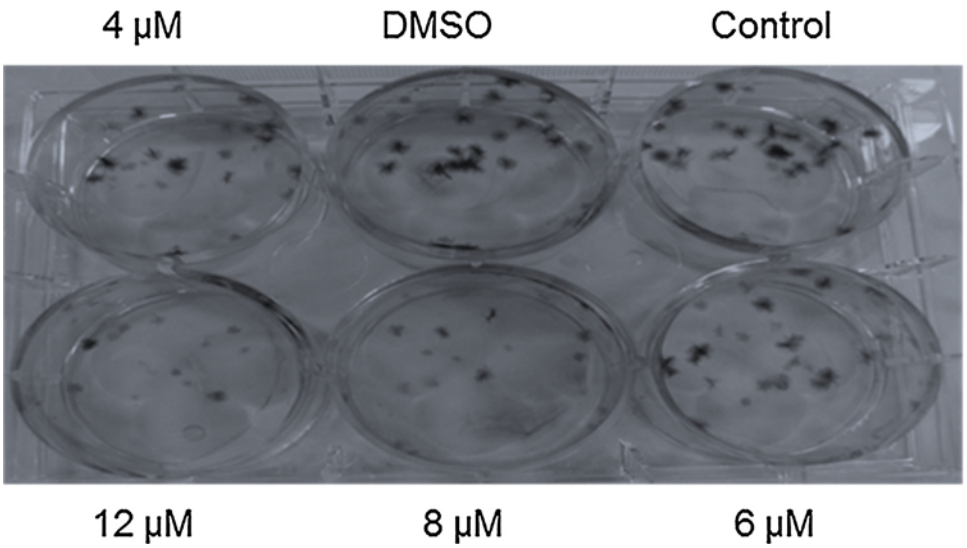

Fig. 4. Effect of nitidine chloride (NC) on C6 cells proliferation. NC was shown to arrest colony formation at effective concentration of 8 µM.

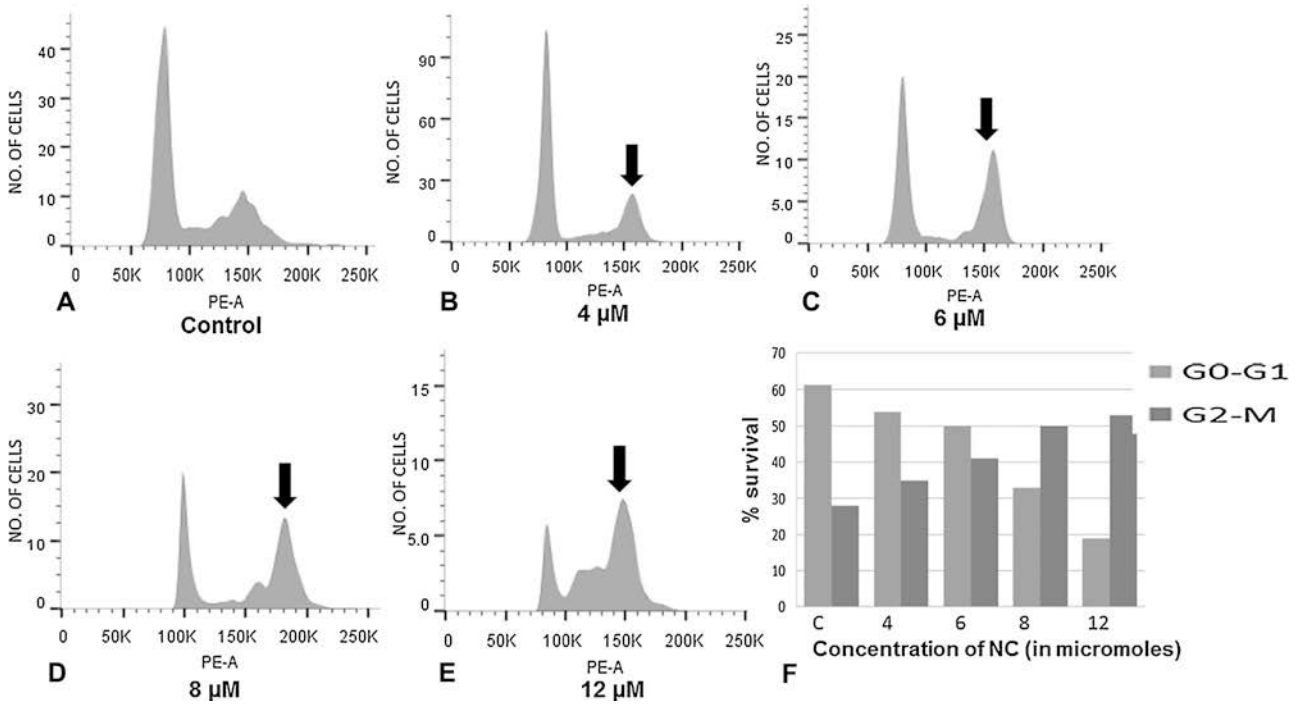

Fig. 5. Cell cycle analysis of nitidine chloride (NC) in C6 cell line treated with empty vehicle (DMSO) (A), 4 (B), 6 (C), 8 (D), and 12 μM (E). Cell cycle arrest was observed in G2/M phase of cell cycle (F).

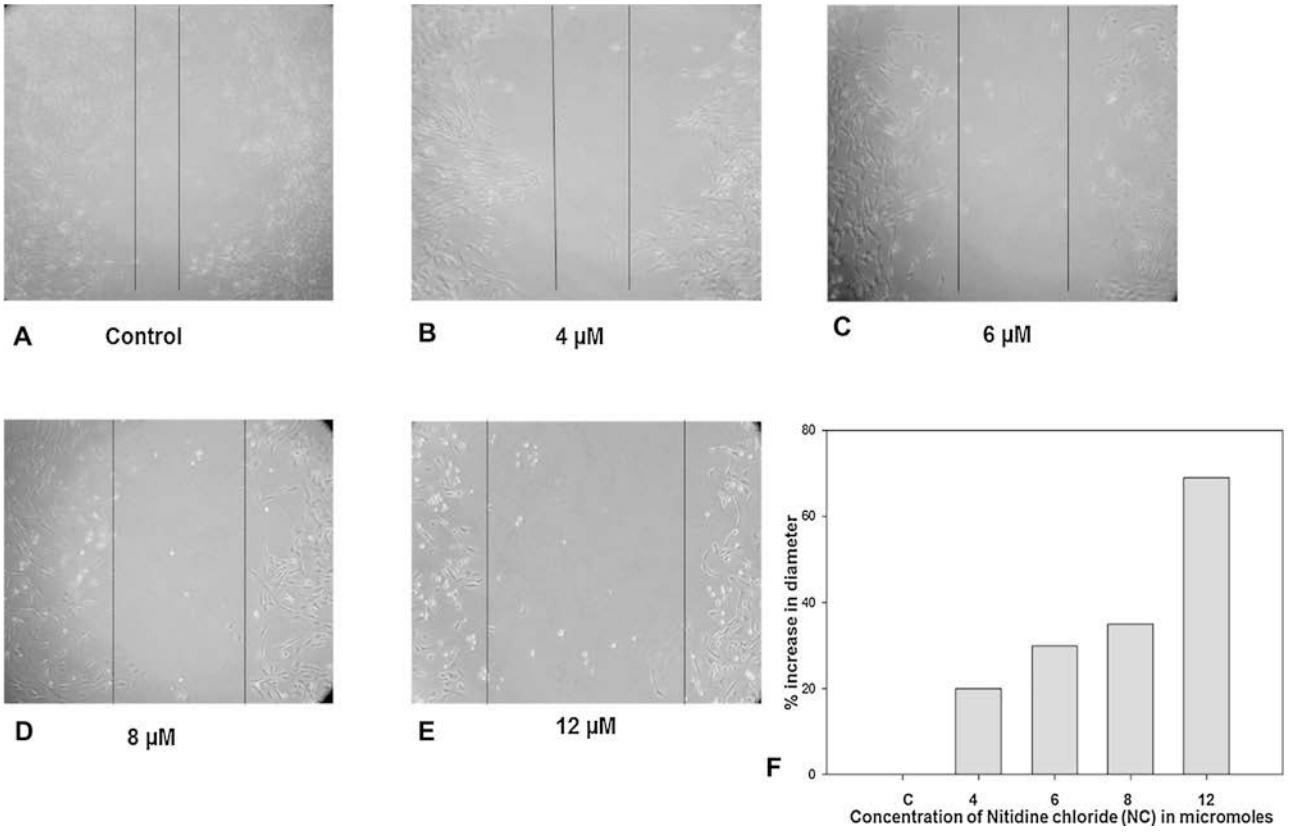

Fig. 6. Wound healing assay for C6 cell line treated with empty vehicle (DMSO) (A), 4 μM (B), 6 μM (C), 8 μM (D), and 12 μM (E). Diameter of wound was calculated as percentage increase compared with control. It is evident that the diameter of wound gradually increased with effective nitidine chloride (NC) concentration (F).

Results

NC inhibits glioma cell proliferation

In the present study, we have investigated the anti proliferative effect of NC on C6 and U87 cell lines using MTT assay. NC displayed dose-dependent inhibition of cell growth in U87 (Fig. 1) and C6 (Fig. 2) cell lines. In U87 cell lines, the IC50 value appeared to be around 10 μM while in C6 cell line, it was around 8 μM (Fig. 3). Furthermore, we have seen the effect of NC on colony formation in C6 cell line (Fig. 4). The effective concentrations of MTT assay were selected. Here, it was clear that NC displayed IC50 value at 8 μM. These results were consistent with the initial MTT assay.

NC-induced G2/M cell cycle arrest in C6 glioma cell line

The effect of NC on cell cycle arrest was assessed in C6 glioma cell line by flow cytometry. C6 cells were treated with four varied concentrations (4, 6, 8, and 12 μM) of NC. The control cells were treated with empty vehicle (DMSO). It is clear from Fig. 5(A–E) that NC-induced cell arrests in G2/M phase of cell cycle in a dose-dependent way that was accompanied by a decrease in G0/G1 phase (Fig. 5-F). In C6 cells, the percentage of cells in G2/M phase was increased from 28% to 52%. In cells treated with 8 μM of NC, 50% of cells were found to be arrested in G2/M phase of cell cycle. The results suggested that NC arrests C6 cells in G2/M phase of cell cycle.

NC inhibits wound healing of C6 cells

It is clear from Fig. 6 that NC treatment resulted in inhibition of wound healing in C6 cells in a dose-dependent manner (A–E). Cells in control panel were treated with empty vehicle (DMSO). The percentage increase in diameter was calculated as compared with the control. Cell treated with 6 and 8 μM of NC showed 30% and 35% increase in diameter, respectively, as compared to control. Cells treated with 4 μM were found to increase 30% in diameter. Cells treated with 12 μM were found to display nearly 70% increase in diameter as compared to control (F).

NC induces apoptosis through alteration of caspase and Gsk3 beta

Western blot experiments (Fig. 7A) demonstrated that NC-induced cell death in caspase 3 dependent ways. C6 cells were treated with 4, 8 and 12 μg of NC. Cell death was found to increase with dose and was statistically significant ($p < 0.05$) in 8 and 12 μg treatment module as compared to control (Fig. 8C). Conversely, we found that pGsk3 expression diminished with progressive NC treatment. Decrease in pGsk3 beta in C6 cells treated with 12 μM of NC was statistically significant ($p < 0.05$) as compared to control for 4 and 8 μM (Fig. 8D). Further, we also found that cleaved PARP and cleaved caspase 7 were overexpressed on NC treatment. Increased expression of cleaved PARP and caspase 7 in C6 cells treated with 12 μM of NC was found to be statistically significant as compared to empty vehicle (DMSO) treated cells (Fig. 8A and B). Furthermore, we also have assessed the effect of NC on pDok2 expression, which has been recently reported as a possible therapeutic target for human malignant glioma. It was found that NC inhibited pDok2 protein expression in U87 malignant human glioblastoma cell line. In addition, the decrease in pDok2 protein expression in U87 cells treated with 12 μM of NC was statistically significant as compared to empty vehicle (DMSO) treated control U87 cells (Fig. 8: E).

Discussion

Traditional herbs are being widely used to extract bioactive compounds including alkaloids, terpenoids, and flavonoids. Recently, researchers have focused on its possible use for anticancer treatment. Accumulating literature have amply justified the anti-cancer activities of NC on various malignancies including renal cancer, gastric adenocarcinoma, and lung adenocarcinoma [10–12]. NC has been shown to exert mTOR-dependent cell arrest activity in U87 human glioma cells [9]. However, the activity of NC has not been reported in rat glioma cells as they are most widely used in preclinical investigations. Our results shed light on anti-cancer activities of NC on rat glioma cells (C6). Further, Dok2 has been reviewed as a potential target in colorectal and gastric carcinoma [13,14]. Recently, we have shown that pDok2 protein significantly expressed itself in human glioblastoma multiforme and is correlated with poor prognosis in clinical cases [15]. We have also reported that NC inhibits Dok2 expression in a dose dependent-way and can be viewed as a therapeutic target.

In this study, we showed that NC inhibits glioma cells proliferation in a dose-dependent manner. The IC50 value of 8 μM was found to be consistent as validated by MTT (Figs. 1–3) and clonogenic assays (Fig. 4). We have accessed the cell death on NC treatment with propidium iodide (PI) staining by flow cytometry. It was evident from the results that NC treatment arrests the C6 cells in G2/M phase of cell cycle. Cells treated with NC (4, 6, 8 and 12 μM) showed a progressive decrease in the cell population in G0-G1 phase of cell cycle while there was an increase in the percentage of cells in G2/M phase of the cell cycle (Fig. 5). The present results have been supported by similar studies on breast cancer cell lines [19]. NC was also found to inhibit wound healing at effective concentrations of 8 and 12 μM. Cells treated

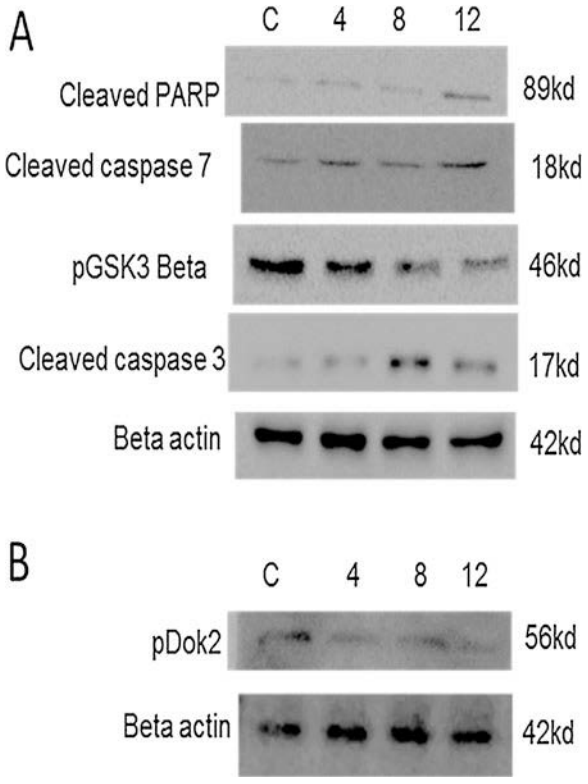

Fig. 7. Western blot analysis (A) for the effect of nitidine chloride (NC) on cleaved PARP, cleaved caspase 7, pGSK3 beta, and cleaved caspase 3 in C6 cell line. (B) Represents NC-mediated down regulation of pDok2 expression in U87 human malignant glioma cell line.

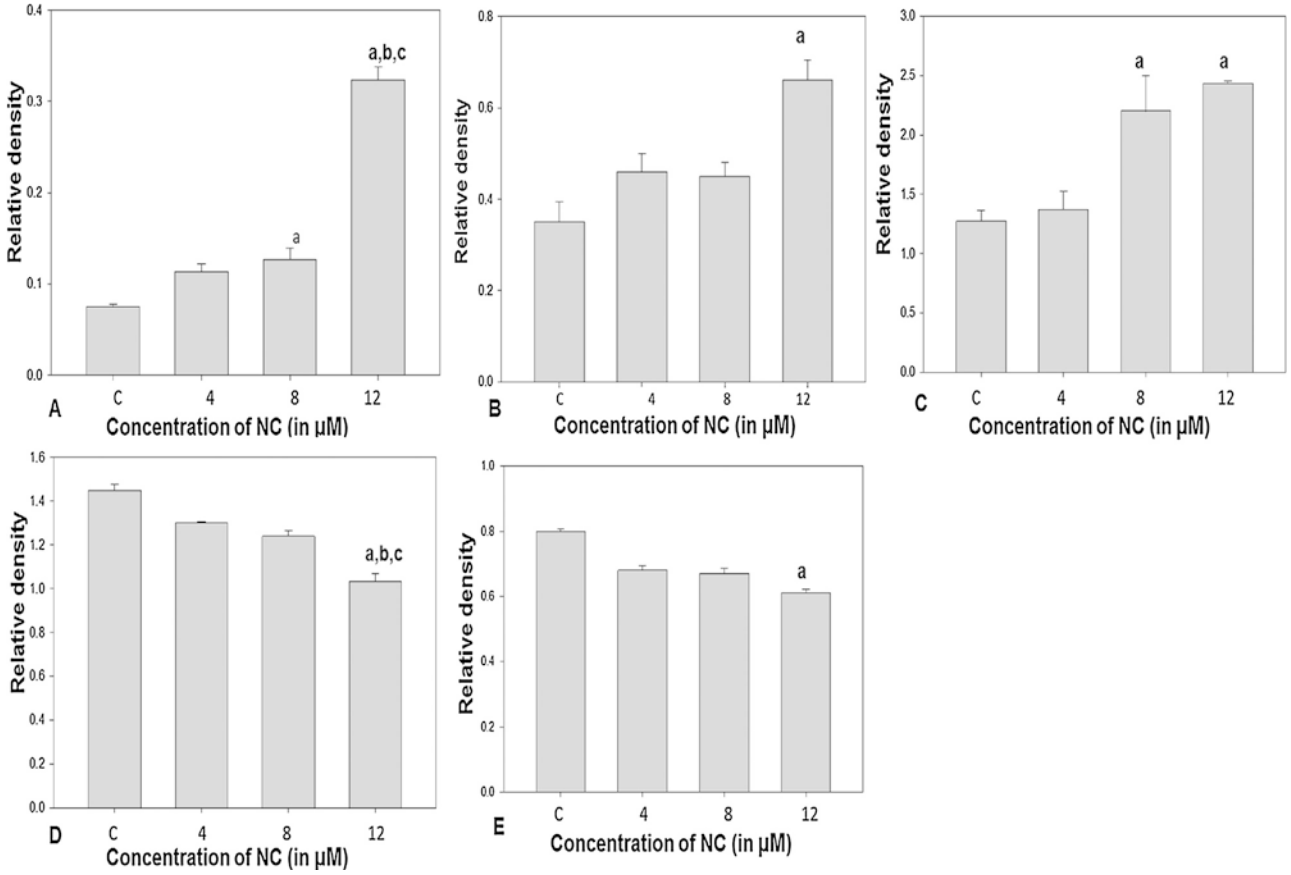

Fig. 8. Densitometric analysis for cleaved PARP (A), cleaved caspase 7 (B), caspase 3 (C), pGSK3 beta (D) in nitidine chloride (NC) treated C6 cell line. pDok2 was found to be down regulated in NC treated U87 cells. The degree of down regulation in U87 cells treated with 12 μM of NC was statistically significant as compared with control (a) (E). Cleaved caspase 3 expression was found to increase as a function of NC treatment while Gsk3 beta expression was decreased with NC treatment. Increased expression of caspase 3 for 8 and 12 μM was statistically significant ($p < 0.05$) as compared with control (a). Decreased expression of Gsk3 beta for 12 μM was statistically significant ($p < 0.05$) as compared with control (a), 4 (b), and 8 (c) μM (D). Overall, the significance of expression in cells treated with 12 μM of NC was evaluated as compared with empty Vehicle (DMSO) (a), 4 μM (b) and 8 μM (c) of NC.

with 12 μM of NC were found to show 70% increase in diameter as compared to control (Fig. 6). The present results have been supported by the existing literature [12]. Here, NC was reported to arrest MCF-7 and MDA-MB-231 cells in G2/M phase of the cell cycle. This arrest was accompanied by a decreased number of cells in S phase of cell cycle. Western blotting results demonstrated that NC treatment induced caspase 3 dependent cell death at previously verified concentrations. The upregulation of cleaved caspase 7 in C6 cells at 12 μM concentration was found to be statistically significant as compared to C6 cells treated with empty vehicle ($p = 0.019$). It was found that NC inhibits Gsk3 beta protein expression. The degree of Gsk3 beta suppression in cells treated with 12 μM of NC was statistically significant as compared to control and cells treated with 4 and 8 μM of NC (Figs. 7 and 8). Recently, NC has been shown to inhibit akt, a component of Gsk3 beta cascade, and has been an active candidate for possible therapeutic intervention [9]. NC was also found to exert an inhibitory effect on cleaved PARP and caspase 7 proteins, which are reported to be deregulated in glioma biology [15–18]. We observed the cleaved PARP protein expression that progressively increased from control to NC-treated (4, 8, and 12 μM) cells. The degree of upregulation in glioma cells treated with 12 μM of NC was statistically significant as compared to control and glioma cells treated with 4 and 8 μM of NC ($p < 0.001$). Similarly, we observed

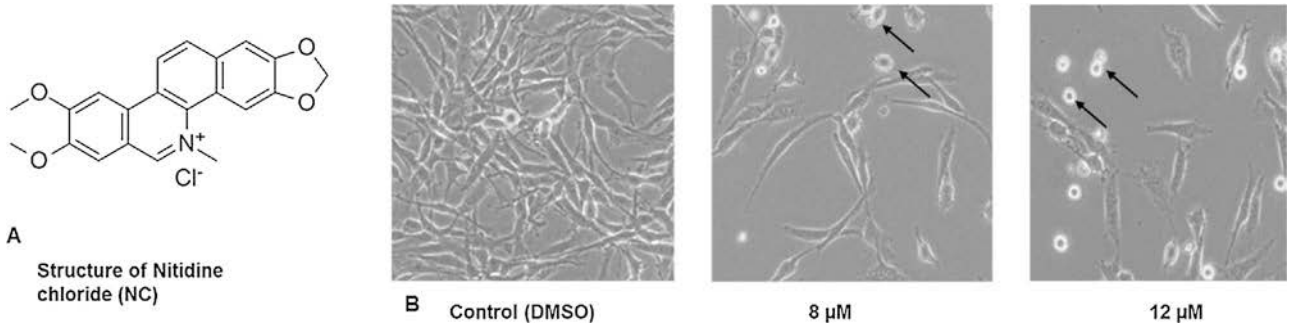

Fig. 9. Structure of nitidine chloride (NC) (A) and morphological appearance (B) of C6 cells on NC treatment. Cells with altered morphology have been marked by an arrow.

cleaved caspase 3 protein expression that was significantly upregulated in C6 glioma cells treated with 8 and 12 μM as compared to control.

In summary, our findings imply that NC alters cell morphology (Fig. 9), exerts arrest of cell cycle and colony formation in C6 cells, and is supplemented with its inhibitory effect on wound healing activity. Further investigations are necessary to ascertain the downstream targets to uncover its therapeutic potential.

Conflict of interest

Authors declare that there are no conflicts of interest.

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