Norcantharidin blocks Wnt/β-catenin signaling via promoter demethylation of WIF-1 in glioma

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Abstract. Glioma is one of the most common primary intracranial tumors, and the prognosis is poor even though much treatment management is employed. Wnt/β-catenin signaling has been reported to be associated with glioma. Norcantharidin (NCTD) is the demethylated analog of cantharidin isolated from blister beetles, and it is reported to possess anticancer activity but less nephrotoxicity than cantharidin. Accordingly, we aimed to investigate NCTD as an anti-neoplastic drug that inhibits the Wnt/β-catenin pathway via promoter demethylation of Wnt inhibitory factor-1 (WIF-1) in glioma growth *in vitro*. In the present study, we report that NCTD inhibited cell proliferation, induced apoptosis and cell cycle arrest, and suppressed cell migration and invasion *in vitro*. Moreover, we observed that the expression levels of WIF-1 mRNA and protein in the NCTD-treated cells were increased significantly compared with these levels in the negative control (NC) cells. Promoter demethylation was observed in the NCTD-treated cells. In contrast, aberrant methylation was observed in the NC cells. Additionally, more investigation revealed that NCTD suppressed activity of Wnt/β-catenin signaling and transcription of β-catenin/TCF-4. Furthermore, the expression of apoptosis-related proteins Bcl-2 and cleaved caspase-3 indicated significant cell apoptosis. We provide initial evidence that NCTD reactivates WIF-1 from a methylation state, and downregulates canonical Wnt/β-catenin signaling. Our findings revealed that NCTD is effective for glioma *in vitro* and may be a new therapeutic option *in vivo*.

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Abbreviations: NC, negative control; WIF-1, Wnt inhibitory factor-1; NCTD, norcantharidin; DNMT1, DNA methyltransferase-1

Key words: norcantharidin, Wnt inhibitory factor-1, Wnt/β-catenin signaling, glioma

Introduction

Glioma is one of the most common malignant tumors in the brain, with a median patient survival of 12 months (1). Despite the rapid progress of new insights and technology in therapy and nursing care, the poor prognosis has persisted during the past few decades, in particular in glioblastoma cases (1-5). Therefore, it is essential to investigate the mechanisms involved in glioma development and progression and to identify a cure.

Wnt/β-catenin signaling has been proven to be associated with various disease pathologies, especially in gliomagenesis. The pathway activates downstream targets and thereby regulates many biological processes through a complex of β-catenin and T cell factor/lymphoid-enhancer factor 1 (TCF/LEF-1) family. Wnt stabilizes cytosolic β-catenin, which then binds to TCF/LEF-1 in the nucleus and recruits transcription factors Brg1 and CREB-binding protein to initiate Wnt-targeted gene expression (6). Wnt inhibitory factor (WIF) inhibits Wnt signaling by direct binding to Wnt molecules, acting as an important antagonist. Wnt inhibitory factor-1 (WIF-1) silencing, due to promoter hypermethylation, has been observed in glioma (7-9).

Norcantharidin (NCTD) is the demethylated analog of cantharidin isolated from blister beetles. NCTD has been reported to possess anticancer activity but less nephrotoxicity than cantharidin (10). Investigators have reported the anticancer effect of NCTD against human glioma cells. Yet, the demethylation of NCTD has not been previously studied. Herein, we hypothesized that NCTD may be used as an effective and nontoxic demethylating agent of the WIF-1 promoter.

In the present study, we showed that NCTD suppressed Wnt/β-catenin signaling via demethylation of the WIF-1 promoter in glioma cell lines. The results indicated that NCTD blocks Wnt/ β-catenin signaling, with impaired nuclear translocation of β-catenin. Our results establish an important role for NCTD in the treatment of brain gliomas, since it can cross the blood-brain barrier. Thus, this may enhance the potential use of NCTD to relieve patients suffering from gliomas.

Materials and methods

Cell culture, culture conditions and reagents. The human LN229 and U251 cell lines were purchased from the Institute of Biochemistry and Cell Biology, the Chinese Academy of

Sciences. All cells were maintained at 37˚C in a 5% CO_2 incubator in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Invitrogen). NCTD was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell proliferation and MTS assays. Cells were plated in 96-well plates at a density of 5,000 cells/well. Cells were allowed to attach overnight in growth medium. After 24 h, the cells were treated with NCTD. After incubation for 72 h, cellular proliferation was measured using the MTS assay and absorbance was measured at 490 nm. Proliferation data are presented as mean ± SD.

Cell cycle assay. For cell cycle analysis by flow cytometry, NCTD-treated and NC cells in the log phase of growth were harvested, washed with phosphate-buffered saline (PBS), fixed with 90% ethanol overnight at 4˚C, and then incubated with RNase at 37˚C for 30 min. Nuclei of the cells were stained with propidium iodide for an additional 30 min. A total of 20,000 nuclei were examined in a FACSCalibur flow cytometer (Becton-Dickinson), and DNA histograms were analyzed using Modifit software. Three independent experiments were performed, and the data are presented as the mean ± SD.

Apoptosis assay. Forty-eight hours after treatment with NCTD and PBS, apoptosis in the cultured cells was evaluated using Annexin V labeling. For the Annexin V assay, an Annexin V-FITC-labeled Apoptosis Detection kit (Abcam) was used according to the manufacturer's instructions. Three independent experiments were performed, and the data are presented as the mean ± SD.

Transwell invasion and migration assays. Transwell invasion assay filters (Costar) were coated with Matrigel (3.9 mg/ml, 60-80 μl) on the upper surface of a polycarbonate membrane (diameter 6.5 mm and pore size 8 μm). After incubating at 37˚C for 30 min, the Matrigel had solidified and served as the extracellular matrix for the tumor cell invasion analysis. The Transwell migration assay was carried out without the presentation of Matrigel on the upper surface of the polycarbonate membrane. A total of 600 μl of conditioned medium derived from the tumor cells was used as a source of chemoattractant and was placed in the bottom compartment of the chamber. Harvested cells ($5x10^4$) in 100 μl of serum-free DMEM were added into the upper compartment of the chamber. After 24 h of incubation at 37˚C with 5% CO_2, the medium was removed from the upper chamber. The non-invaded cells on the upper side of the chamber were scraped off with a cotton swab. The cells that had invaded from Matrigel into the pores of the inserted filter were fixed with 100% methanol, and stained with crystal violet. The number of cells invading through the Matrigel was counted using three randomly selected visual fields from the central and peripheral portions of the filter by an inverted microscope at x200 magnification. Each assay was repeated three times.

RNA extraction and quantitative real-time polymerase chain reaction. Total RNA was isolated using TRIzol reagent (Invitrogen) and cDNA was synthesized using the High Capacity cDNA Archive kit (Applied Biosystems) according to the manufacturer's instructions.

WIF-1 and cyclin B1 mRNA were quantified by qRT-PCR using SYBR Premix Ex Taq (Takara Bio, Inc.). The primers were: WIF-1 forward, 5'-CCGAAATGGAGGCTTTTGTA-3' and reverse, 5'-TGGTTGAGCAGTTTGCTTTG-3'; cyclin B1 forward, 5'-GCAACCTCCAAGCCCGGACTG-3' and reverse, 5'-GCAACCTCCAAGCCCGGACTG-3'.

The PCR conditions included an initial denaturation step of 95˚C for 2 min, followed by 35 cycles of 95˚C for 10 sec, 56˚C for 20 sec and 72˚C for 20 sec, and a final elongation step of 72˚C for 10 min. All qRT-PCRs were performed in duplicate. Quantitation relative to the endogenous control was carried out using Applied Biosystems 7500 Fast System SDS software.

Methylation-specific PCR (MS-PCR). Genomic DNA from the cell lines and normal lung tissue was extracted using the DNeasy kit (Qiagen), according to the manufacturer's instructions. Bisulfite modification of genomic DNA was performed using a methylation kit (EZ DNA Methylation kit; Zymo Research, Orange, CA, USA). Bisulfite-treated genomic DNA was amplified using either a methylation-specific or an unmethylation-specific primer set. HotStarTaq DNA polymerase (Qiagen) was used in the experiments.

Sequences of the methylation-specific primers were 5'-GGGCGTTTTATTGGGCGTAT-3' (forward) and 5'-AAA CCAACAATCAACGAAC-3' (reverse). Sequences of the unmethylation-specific primers were 5'-GGGTGTTTTATTG GGTGTAT-3' (forward) and 5'-AAACCAACAATCAACAA AAC-3' (reverse).

Western blotting. Extraction of proteins from the cultured cells was followed by immunoblotting with the relevant antibodies. Each experiment was repeated at least three times.

Immunofluorescence staining assay. Immunofluorescence staining was conducted with LN229 and U251 cells cultured on coverslips. The cells were fixed in 4% paraformaldehyde and permeabilized for 10 min in buffer containing 0.1% Triton X-100. The relevant antibodies were then added at the dilutions recommended by the manufacturers. DAPI reagent was used to stain the glioma cell nuclei, and the cells were visualized using an FV-1000 laser-scanning confocal microscope and analyzed using IPP5.1 (Olympus, Tokyo, Japan).

Statistical analysis. Quantitative variables are expressed as mean ± SD and analyzed by one-way analysis of variance (ANOVA) and the Student's t-test. All differences were considered to be statistically significant at the level of $P<0.05$. Statistics were performed using the SPSS Graduate Pack version 11.0 statistical software (SPSS, Inc., Chicago, IL, USA).

Results

NCTD suppresses the growth of LN229 and U251 cell lines. The LN229 and U251 cells were exposed to 0, 3.75, 7.5, 15, 30, 60 and 120 μM NCTD and PBS for 48 h. The results indicated that the cellular viability decreased with increasing NCTD

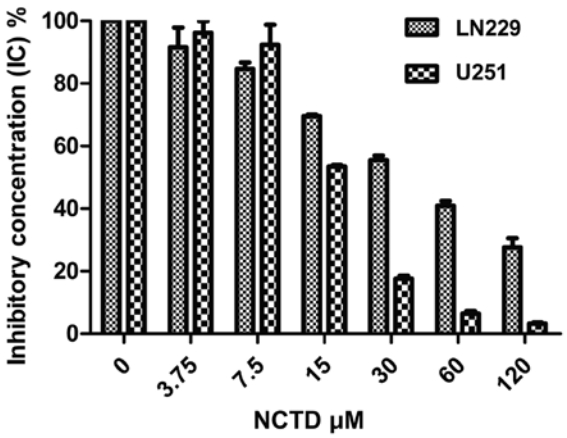


Figure 1. Effects NCTD on the proliferation of LN229 and U251 cells. Cells were treated with NCTD (3.75-120 μM) and PBS, and incubated for 24 h. Proliferation was assessed by MTS assay. Experiments were performed independently at least 3 times. Data are the mean ± SD. NCTD, norcantharidin.

concentrations (Fig. 1). The 50% growth inhibition (IC_{50}) of NCTD was 44 μM for LN229 and 10 μM for U251 cells after 48 h, respectively.

NCTD increases cell cycle arrest at the G2 phase in the LN229 and U251 cell lines. To further characterize the growth arrest, LN229 and U251 cell lines were treated with 44 and 10 μM NCTD, and after 48 h they were subjected to propidium iodide-staining and FACS analysis. Treatment with NCTD resulted in accumulation of cells in the G2 phase, with ~42.33±0.514% of NCTD-treated LN229 and 28.28±2.74% of NCTD-treated U251 cells in the G2 phase compared with 9.09±0.09% PBS-treated LN229 and 16.80±0.57% PBS-treated U251 cells (Fig. 2A) (P<0.05). Cyclin B1 level was decreased in the cell lines, as shown using western blotting (Fig. 3). These analyses indicate that NCTD induced cell cycle arrest at the G2 phase.

NCTD increases cell apoptosis in the LN229 and U251 cell lines. NCTD also inhibited glioma cell survival. As shown in Fig. 2B, compared with the NC groups (4.47±1.13 and 4.55±2.11%) in the LN229 and U251 cells, the treatment of NCTD caused a significant increase in apoptotic death (26.40± and 32.00±5.00%) (P<0.05). The Bcl-2 protein level decreased and the cleaved caspase-3 protein level increased in the NCTD-treated cell lines, as shown using western blotting (Fig. 3).

The intrinsic pathway of apoptosis is controlled by Bcl-2 family proteins, and cell death depends on the balance between pro-apoptotic (Bax) and anti-apoptotic (Bcl-2, Bcl-xl and Mcl-1) proteins. We also investigated the treatment effect on such proteins. As shown in Fig. 3, treatment with NCTD had some effect on Bax expression. However, treatment with NCTD resulted in a significant downregulation of Bcl-2. Cleaved caspase-3 release was detected by western blot analysis after treatment of NCTD. The activity of pro-caspase-3 cleaved to caspase-3 increased after treatment of NCTD. Together, this analysis demonstrated that NCTD induced downregulation of Bcl-2 as previously reported but had little effect on Bax. Activation of cleaved caspase-3 indicated that the compound induced the intrinsic and extrinsic apoptotic pathways.

NCTD suppresses the invasion and migration of the LN229 and U251 cell lines. To test whether NCTD regulates tumor cell invasion and migration, Transwell assays were performed. The assays showed that NCTD markedly inhibited Transwell invasion and migration of glioma cells (Fig. 2C and D). In regards to the LN229 cells, the number of cells invading through the Matrigel in the NCTD group was 38.30±2.87 which was significantly decreased compared with the number of invading cells in the NC group (110.00±8.20) (P<0.05). Regarding the U251 cells, the invasive activity was also inhibited in the NCTD group (41.3±3.30), when compared with that of the NC group (90.67±8.16) (P<0.05). Regarding the LN229 cells, the number of cells migrating through the polycarbonate membrane in the NCTD group was 55.67±4.19, significantly decreased when compared with the number of invading cells in the NC group (157.00±2.94) (P<0.05). Regarding the U251 cells, the invasive activity was also inhibited in the NCTD group (47.33±1.70), compared with that of the NC group (95.00±4.08) (P<0.05). The above data showed that NCTD suppressed the invasion and migration of the human glioma cells.

Expression of the mRNA transcript, promoter methylation and protein expression of WIF-1 in the LN229 and U251 cell lines. qRT-PCR assay was also performed to analyze the expression of WIF-1 at the transcription level. We found that the WIF-1 transcript was downregulated in the LN229 and U251 cell lines in the NC group. In contrast, it was upregulated in the NCTD-treated cells (Fig. 4A). The results showed that WIF-1 expression in the LN229 and U251 cell lines in the NC group (10.00±0.00) was significantly lower compared with the expression level in NCTD-treated cells (50.23±5.00 and 61.00±6.56) (P<0.05). To examine whether the methylation status of the promoter correlates with the expression of WIF-1, methylation-specific PCR was carried out (Fig. 4B). Promoter demethylation was observed in the NCTD-treated cells. In contrast, aberrant methylation was observed in the NC group. To detect the expression level of WIF-1, western blotting was performed in the LN229 and U251 cell lines (Fig. 4C). The level of WIF-1 expression was significantly lower in the LN229 and U251 cell lines in the NC group than those levels in the NCTD-treated cells, but expression of DNA methyltransferase-1 (DNMT1) was not influenced (Fig. 4C).

NCTD decreases cyclin B1 mRNA expression. qRT-PCR results determined that the relative expression level of cyclin B1 in the NCTD-treated LN229 cells was 20.40±5.43% (P<0.05), and 35.54±5.99% in the NCTD-treated U251 cells (P<0.05), compared with the NC groups, respectively (Fig. 4D).

NCTD inhibits Wnt/β-catenin signaling through loss of nuclear β-catenin. β-catenin, Wnt-target proteins and their expression in many cell lines have been described. Wnt signaling has been proven to be associated with various disease pathologies. NCTD was reported to act as a negative regulator of Wnt signaling. Western blotting of total protein extracts from the LN229 and U251 cells revealed that the total β-catenin content

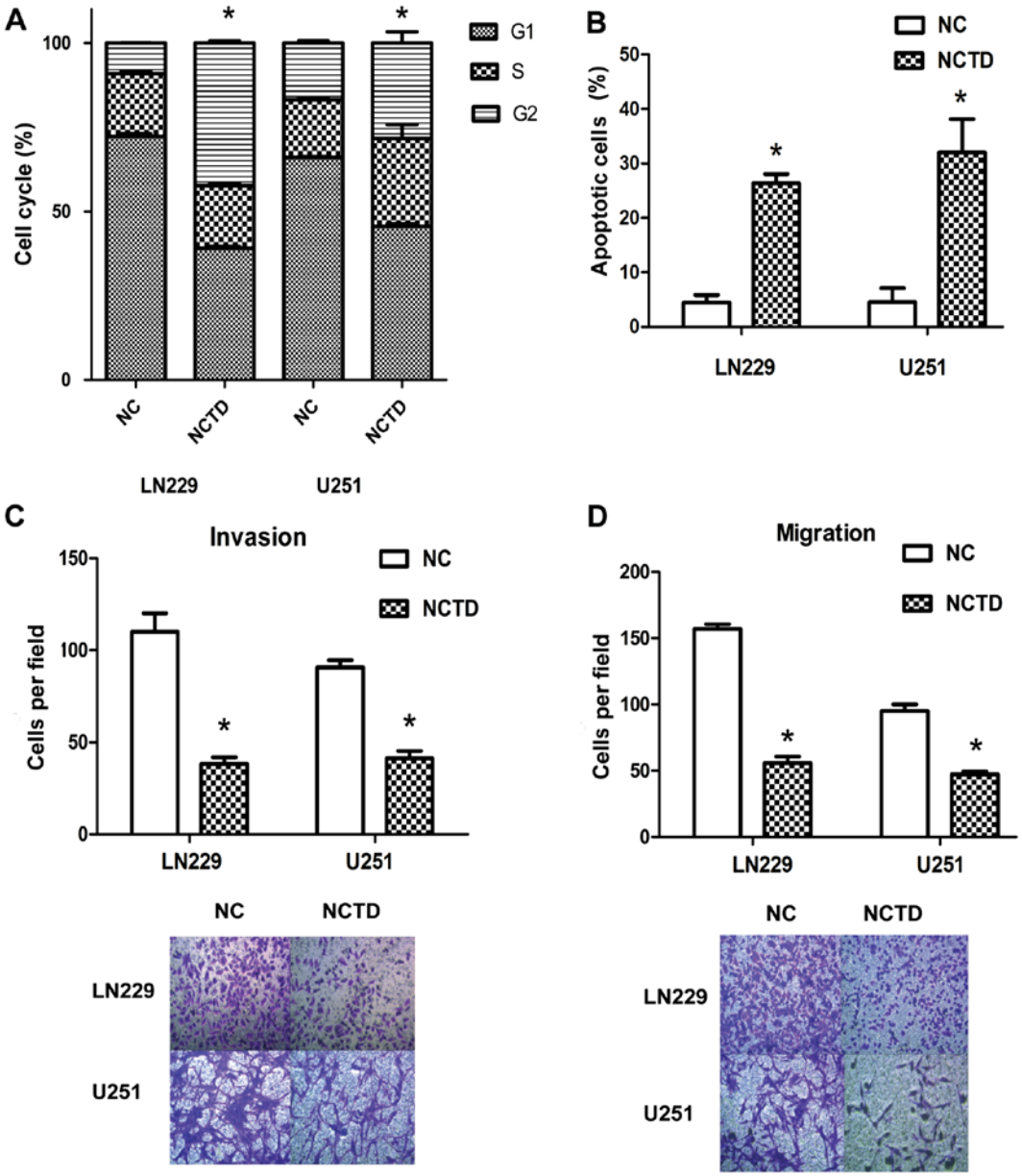
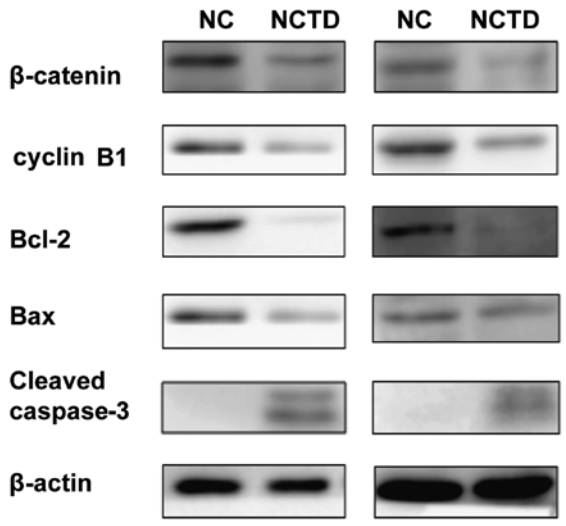


Figure 2. NCTD promotes cell cycle arrest and apoptosis, and suppresses glioma cell invasion and migration. (A and B) NCTD significantly led to G2 phase arrest and apoptosis of the LN229 and U251 cells (*P<0.05), relative to the NC cells. (C and D) NCTD significantly suppressed invasion and migration in both the LN229 and U251 cells (*P<0.05), relative to the NC cells. NCTD, norcantharidin; NC, negative control.



was reduced after treatment with NCTD (Fig. 3). The level of protein cyclin B1, a known Wnt downstream target, was also significantly reduced (Fig. 3). Immunofluorescence assays in the LN229 and U251 cell lines revealed altered nuclear location of β-catenin. After treatment of NCTD, β-catenin was mainly located in the cytoplasm, while β-catenin was mainly located in the nucleus in the NC cells (Fig. 5). This showed that the location of β-catenin in cells shifted from the nucleus to the cytoplasm when the cells were treated with NCTD.

Figure 3. Expression of related proteins after treatment with NCTD. Expression levels of β-catenin and apoptosis-related proteins were altered after treatment of NCTD in the LN229 and U251 cells. Protein levels were determined with western blot analysis using β-actin as an internal control. NCTD, norcantharidin.

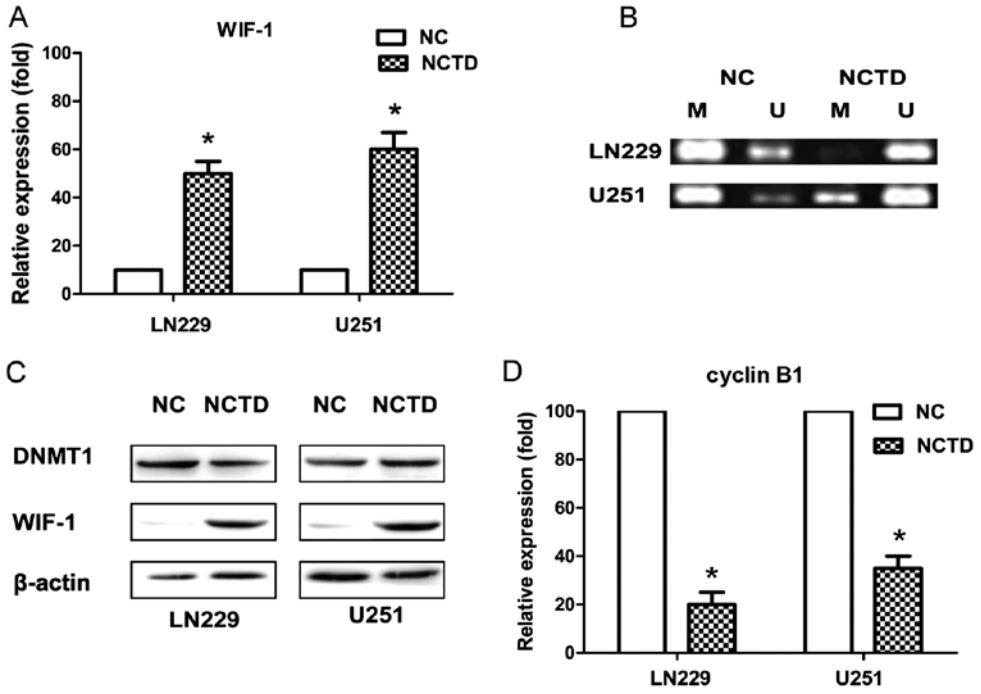


Figure 4. NCTD promotes the expression of WIF-1 and suppresses the expression of cyclin B1. (A) WIF-1 mRNA expression was quantified by qRT-PCR analysis, and the expression was significantly promoted after administration of NCTD in both the LN229 and U251 cells (*P<0.05), relative to the NC group. (B) Methylation-specific PCR (MS-PCR) analysis of the CpG island in the NC and NCTD-treated groups. Bands in lane U indicate the unmethylated DNA product with unmethylation-specific primers. Bands in lane M indicate the methylated DNA product amplified with methylation-specific primers. NCTD significantly promoted unmethylation in both the LN229 and U251 cells, relative to the NC group. (C) Expression of DNMT1 and WIF-1 protein was quantified by western blot analysis. WIF-1 expression was significantly promoted in both the LN229 and U251 cell lines, relative to the NC group. (D) Expression of cyclin B1 mRNA was quantified by qRT-PCR analysis, and the expression was significantly promoted in both the LN229 and U251 cell lines (*P<0.05), relative to the NC group. NCTD, norcantharidin; WIF-1, Wnt inhibitory factor-1; DNMT1, DNA methyltransferase-1; NC, negative control.

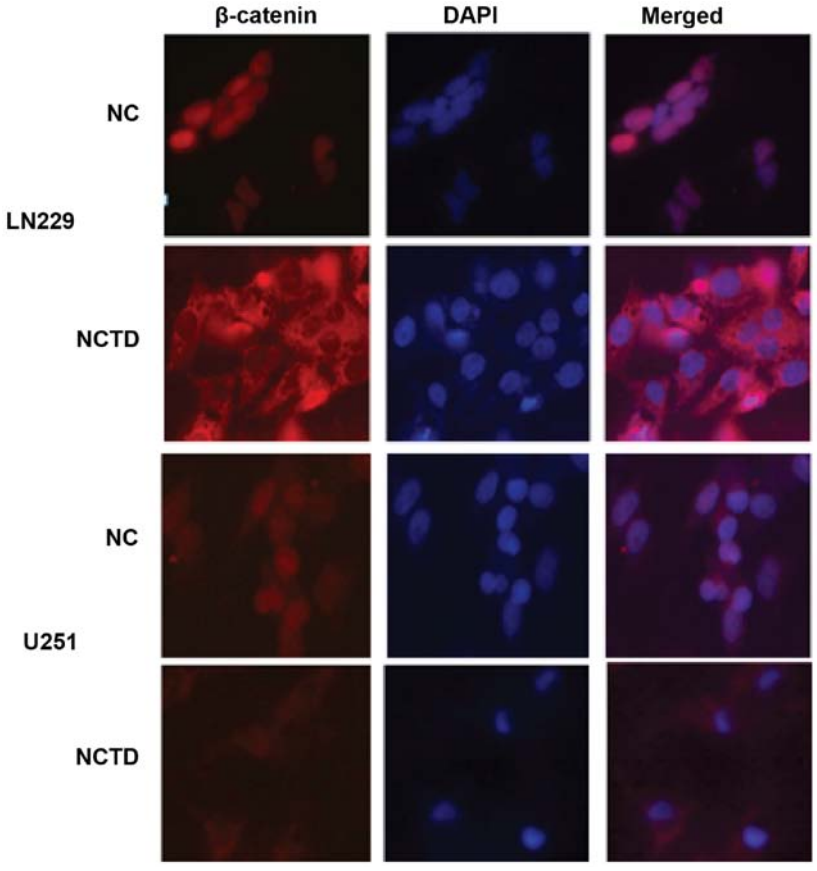


Figure 5. Immunofluorescence assay for β-catenin. The location of β-catenin in cells shifted from the nucleus to the cytoplasm following treatment with NCTD, as compared with the NC group. NCTD, norcantharidin; NC, negative control.

Discussion

Glioma is one of the most frequent types of brain tumor and it is invariably associated with a poor prognosis. Standard treatments include surgery, radiotherapy and chemotherapy (1-4). Prognosis is dismal with an average survival of ~12 months despite the rapid progress of new insights and technology in therapy and nursing care, and the poor prognosis of patients with glioma has remained the same during the past few decades (2-5).

The mechanisms involved in the development and progression depend on many factors, and high-grade gliomas are heterogeneous tumors in both cytology and genetic signatures (5). One major signaling pathway that has been linked to glioma is the Wnt (wingless-type mouse mammary tumor virus integration site family) signaling pathway.

The Wnt signaling pathway activates Wnt-downstream targets and thereby regulates many biological processes through a complex of β-catenin and the TCF/LEF-1 family. Wnt stabilizes cytosolic β-catenin, which then binds to TCF/LEF-1 in the nucleus and recruits transcription factors Brg1 and CREB-binding protein to initiate Wnt-targeted gene expression (6). WIF inhibits Wnt signaling by direct binding to Wnt molecules and acts as an important antagonist (7-9).

Wnt signaling has been proven to be associated with embryonic development (6), tissue renewal and regeneration (11), and various cancer pathologies (12-14), such as cell proliferation, cycle, death invasion and migration (14).

The Wnt/β-catenin signaling pathway is constitutive activated in glioma (15), and aberrant expression of β-catenin in astrocytic gliomas and glioblastoma is linked to a higher tumor grade (16). Some investigators indicate that epigenetic events inactivate the inhibitory components of the Wnt pathway in human cancers. As such, WIF1 in high-grade gliomas is downregulated by promoter hypermethylation (7), and the SFRP1 promoter is commonly hypermethylated in human cancers, including gliomas, among which SFRP1 promoter hypermethylation is most commonly detected in primary glioblastomas (17,18).

Aberrant methylation of promoter regions that silences transcription of genes has been recognized as a mechanism for inactivating tumor-suppressor genes. It occurs at cytosine bases located 5' to a guanosine and so-called CpG dinucleotide short regions of CpG dinucleotides known as CpG islands are found in the proximal promoter region of over half of human genes. WIF-1 silencing may be an early epigenetic carcinogenic event and plays a role in tumor development and progression (7-9).

NCTD is the demethylated analog of cantharidin isolated from blister beetles, and it is reported to possess anticancer activity but less nephrotoxicity than cantharidin (10). There is increasing evidence that NCTD inhibits the proliferation, induces apoptosis (19,20,22-28,30), blocks the cell cycle (20,21,26), suppresses invasion, metastasis (27,29), angiogenesis (27), and possess anticancer activity via MAPK, TnR3, VEGFR2/MEK/ERK, PI3-K/MMPs/Ln-5γ2 signaling, DNA replication, or ROS-mediated mitochondrial dysfunction.

However, few studies have reported the anticancer effect of NCTD against human glioma cells and the early study of anti-glioma focused on Akt and MAPK signaling (31,32). Similarly, NCTD could promote the loss of β-catenin activation and inhibit the proliferation with dominant β-catenin signaling (33-35). These data suggest that NCTD has significant therapeutic potential in cancer via Wnt/β-catenin pathways. DNMT1 could induce gene promoter methylation (36). The demethylation of NCTD has not been previously studied. Herein, we hypothesized that NCTD may be used as an effective and nontoxic demethylating agent of the WIF-1 promoter via suppressing the activity of DNMT1.

In this study, we demonstrated that the expression of the WIF-1 gene was silenced in NC cells via methylation. However, after exposure to NCTD, this epigenetic change was reversed. We also showed that NCTD suppressed nuclear translocation and expression of β-catenin using western blot analysis. These results are consistent with our hypothesis that NCTD may recovery the methylation of WIF-1 by demethylation, and downregulate the Wnt canonical pathway in glioma cell lines.

We provide initial evidence that NCTD reactivates WIF-1 from a methylation state, downregulates the canonical Wnt pathway and establishes an important role for NCTD in the treatment of gliomas, since it can cross the blood-brain barrier. This thus enhances the potential use to relieve patients from suffering from gliomas.

Acknowledgements

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