International Journal of Science and Research (IJSR)
ISSN: 2319-7064
ResearchGate Impact Factor (2018): 0.28 | SJIF (2019): 7.583

Antimitotic Study of TN16 to Tubulin

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Abstract: ***3-(l-anilinoethylidene)-5-benzylpyrrolidine-2, 4-dione (TN16), a synthetic compound, has previously been reported to have anti tumor effect and to inhibit microtubule assembly in vitro. In this study, we found that TN16 inhibited HeLa cell proliferation by depolymerizing the cellular microtubules. HeLa cell proliferation was blocked in mitosis. The present study is consistent with earlier reports of mitosis blocking effects of TN16. By this study, we concluded that TN16 inhibited growth of HeLa cells at IC_{50} of 48±2 nM. The low IC_{50} in nanomolar range is interesting from the point of view of cancer treatment because two major anticancer drugs Vinblastine and Paclitaxel act on Hela cells in nanomolar concentrations. TN16 caused more than 30% mitotic arrest in 200 nM concentration (4x IC_{50}). TN16 depolymerized mitotic microtubules strongly giving rise to aberrant multipolar spindle formation at and above 50 nM concentration. The cells dying through apoptosis in presence of different concentrations of TN16 were also visualized. These data suggest that TN16 inhibited HeLa cell proliferation at mitosis by depolymerizing spindle microtubules.***

Keywords: Tubulin, TN16, HeLa cell line, mitotic arrest

1. Introduction

Microtubules are important cytoskeletal components whose polymerization-depolymerization status or subtle changes in dynamics has serious implications in mitosis, intracellular transport and cellular motility [1]-[4]. Tubulin targeting agents like paclitaxel, vinblastine, vincristine, estramustine have wide spread application in the frontline treatment of cancer chemotherapy although colchicine cannot be used successfully as an antimitotic drug [5]-[8]. Both toxicity and drug resistance are major challenges for successful anticancer treatment as observed in the case of colchicine [9]-[10] identified pharmacophoric attachment points for a number of colchicine site binding agents (CSI) [10]. Some of them bear similarity in structure with colchicine having trimethoxy phenyl ring (A ring) common with colchicine along with some structurally dissimilar drugs. Among the second group of colchicine site binding agents (CSIs), Indanocine. E-7010 and methoxyestradiol are potential candidates [11-13]. They are promising candidates because E7010 and methoxy estradiol are now in clinical trial [14]-[15]. Indanocine also exhibits effectivity against multidrug resistant cell lines [11]. With increasing number of drugs becoming ineffective for cancer treatment, the search for potential anticancer drugs is never over [16]-[17]. TN16 has previously been reported to have anti tumor effect [18] and to inhibit microtubule assembly *in vitro* [19]. TN16 also has been shown to arrest cells in metaphase and hence, used in synchronizing cells to stay at mitotic stage [20]. TN16, being a microtubule assembly inhibitor, is a suitable candidate for further research. So our primary objective with this drug was to study its behavior towards cell lines.

2. Experimental Section

Antibodies and compounds

Mouse monoclonal anti- α tubulin IgG, Mouse monoclonal γ-tubulin, bovine serum albumin (BSA), Hoechst 33258 were purchased from Sigma (St. Louis, MO, USA). Anti Mouse IgG-Alexa 568, FITC-labeled anti-mouse IgG conjugate were purchased from Molecular Probes (Eugene, Oregon, USA). Annexin V-Propidium Iodide (PI) Apoptosis Detection Kit was purchased from Santa Cruz Biotechnology (CA, USA). TN16 was purchased from Calbiochem, USA. All other reagents were of analytical grade.

Cell culture and proliferation assay

HeLa cells were grown in minimal essential media (Himedia, Bangalore, India) supplemented with 10% (v⁄v) fetal bovine serum, 1% antibiotic–antimycotic solution (Himedia,Bangalore, India) containing streptomycin, amphotericin B, and penicillin, and sodium bicarbonate (1.5 mg/mL) at 37^oC in humidified chamber with 5% CO_2 [21]. Briefly, HeLa cells (1 X 10^4 cells/well) were seeded in a polylysine coated 96-well plate and grown for 24 h. 10 mM TN16 sock was made in DMSO. Then, different concentrations of TN16 diluted in media (final concentration of DMSO did not exceed 0.1% of total volume) were added to the wells, and cells were incubated for 24 h. Cells were detached from each well by trypsinization (0.025% trypsin) for 10 min duration. Cells were counted manually using a hemocytometer after staining with 0.4% Trypan Blue and the results were plotted in Origin Pro 7.5 software to determine the IC_{50} value of TN16.

Immuofluorescence Microscopy

The effects of TN16 on microtubules and chromosomes were analyzed by fluorescence microscopy as described recently [22]. Briefly, HeLa cells were grown on glass coverslips at a density of 5 x 10^4 cells/mL in 24-well tissue culture plates for 24 h. The media was then replaced with fresh media containing different concentrations of TN16 and incubated for an additional 24 h. Subsequently, cells were fixed in 3.7 % formaldehyde for 30 min at 37ºC and transferred to ice cold (-20ºC) methanol for 10 min. Nonspecific antibody binding sites were blocked by incubating with 2% BSA in PBS at 37ºC for 30 min. Cells were incubated for 2 h at 25 ºC with mouse monoclonal anti-α-tubulin (1:300 dilution) or mouse monoclonal γ-tubulin (1:2000 dilution). Cells were washed with 2% BSA/PBS for 10 min and incubated with a 1:350 dilution of Alexa-568-labeled anti-mouse IgG antibody or 1:800 dilution of FITC-

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labeled anti-mouse IgG antibody for 1 h. The coverslips were rinsed with PBS and incubated with Hoechst 33258 (1 μg/mL) for 10 min in the dark. The coverslips were mounted in 80% glycerol in PBS containing 8 mg/mL DABCO, (1, 4-diazabicyclo [2.2.2] octane). The coverslips were observed with a Nikon Eclipse TE 2000U microscope (Kanagawa, Japan) by using a 40 X objective. The images were analyzed using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD, USA). The experiment was performed three times.

Mitotic Index

HeLa cells (5 x 10^4 cells/mL) were grown on glass coverslips in a 24-well tissue culture plate for 24 h and then, incubated without or with different concentrations of TN16 for an additional 24 h [22]. Cells were centrifuged in a Labofuge 400R cytospin (Heraeus, Hanau/Germany) for 10 minutes and fixed with 3.7 % formaldehyde for 30 min at 25°C. The fixed cells were washed with phosphate-buffered saline (PBS) and permeabilized with methanol at -20ºC for 10 min and stained with Hoechst 33258 (0.8 μg/mL) for 30 min at 25°C. The mitotic and interphase cells were counted under the Eclipse TE-2000 U microscope (Nikon) using 40X objective. At least 600 cells were counted for each concentration of TN16 and the experiment was repeated three times.

Annexin V/PI staining

Apoptosis was determined by staining cells with annexin V-fluorescein isothiocyanate (FITC) and PI staining [23]. AnnexinV was used to detect early apoptotic cells during apoptotic progression. Briefly, 5×10^4 cells were incubated in 24-well plate with the designated doses of TN 16 for 24 h. The coverslips were washed twice with cold PBS. 500 μl of binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) was added to the coverslips. 1.5 μl of annexin V-FITC solution and 8μl of PI (1 μg/ml) of AnnexinV-PI Apoptosis Detection Kit (Santa Cruz Biotechnology, CA, USA) were added to these cells. The cells were incubated in the dark for 15 min at 25°C. The cells were observed immediately under the Eclipse TE-2000 U microscope (Nikon) using 40X objective. The experiment was performed three times.

3. Results

TN16 inhibited HeLa cell proliferation

TN16 (Figure 1) inhibited the proliferation of HeLa cells in a concentration dependent fashion with an IC_{50} value of about 48 ± 2 nM (Figure 2). For example, 20 and 70 nM TN16 inhibited HeLa cell proliferation by 21.6% and 78%, respectively. The effects of TN16 on the mitotic index were examined over a range of TN16 concentrations. The inhibition of proliferation of HeLa cells was accompanied by cell cycle arrest at mitosis. The mitotic index was 3.3 ± 0.6 % in the absence of TN16; 16.4 ± 3.8%, 29.6 ± 3.8%, and 33.8 ± 4.9% in the presence of 50, 100 and 200 nM TN16 respectively, supporting the previous observation that TN 16 causes metaphase arrest.

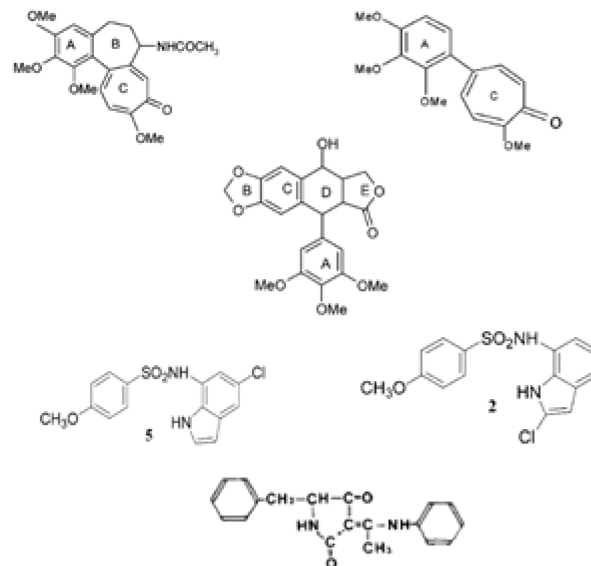

Figure 1: Structure of drugs. (A) Colchicine, (B) AC, (C) Podophyllotoxin, (D) Sulfonamide drug 2, (E) Sulfonamide drug, (F) TN 16

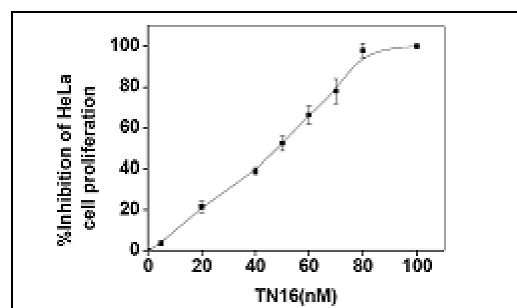

Figure 2: Inhibition of HeLa cell proliferation by TN16

HeLa cells were treated with various concentrations of TN16 (0-200 nM) for 24 h and percent inhibition of proliferation was calculated by counting cells. IC_{50} of TN16 in HeLa cell is 48±2 nM. Data are the average of three independent experiments, presented with ±S.D.

TN16 depolymerized HeLa cell microtubules and disorganized mitotic chromosomes

The effects of TN16 on the interphase and mitotic microtubules are shown in Figure 3 & 4, respectively.

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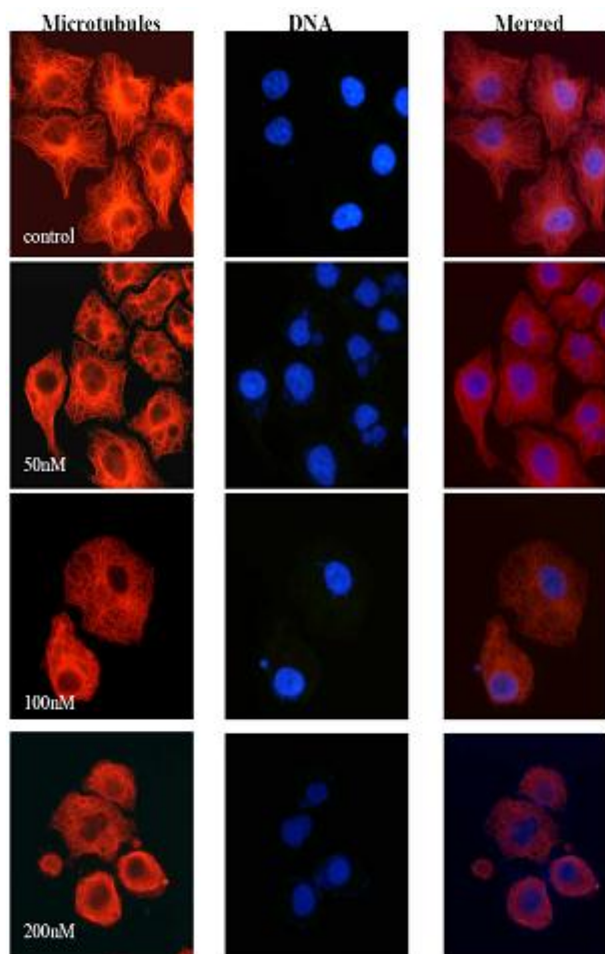

Figure 3: Effects of TN16 on interphase microtubules and chromosome organization

HeLa cells were incubated with indicated concentrations of TN16 for 24 h. Microtubules (red) and chromosomes (blue) were analyzed as described in Materials & Methods.

Control (vehicle treated) interphase microtubules in HeLa cells were found to be well-spread, peripheral microtubules were distinctly visible (Figure 3). TN16 induced depolymerization of interphase microtubules in a concentration dependent fashion. At 50 nM depolymerization was not marked, but at 100 and 200 nM microtubule network was found to be strongly diminished as compared to that of the control cells (Figure 3). With exposure to 200 nM TN16, microtubules were significantly depolymerized near the cell periphery (Figure 3).

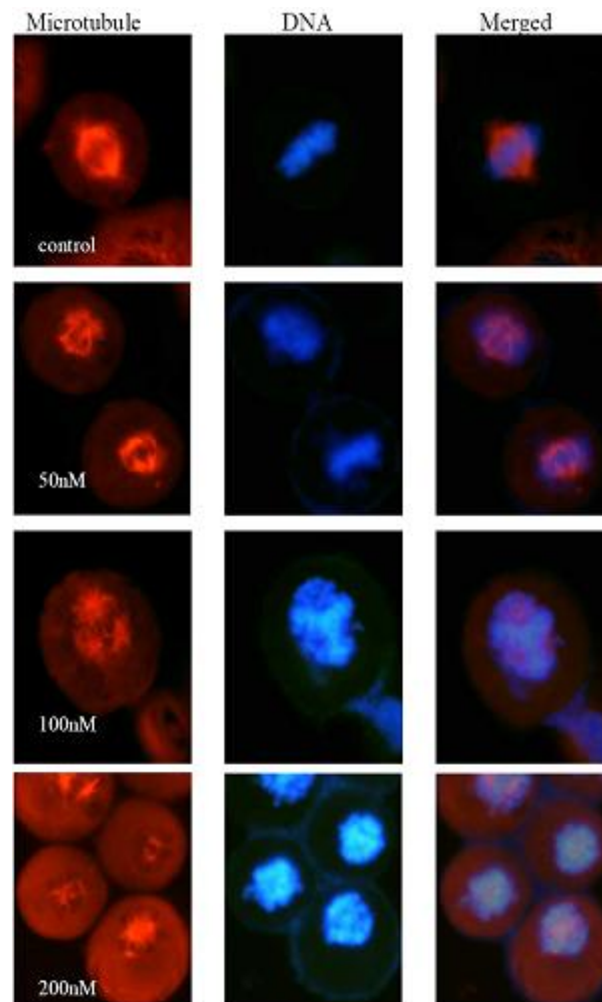

Figure 4: Effects of TN16 on spindle microtubule and chromosome organization. HeLa cells were incubated with indicated concentrations of TN16 for 24 h. Microtubules (red) and chromosomes (blue) were analyzed as described in Materials & Methods.

Control mitotic cells were round with normal bipolar mitotic spindles; chromosomes were properly aligned at the metaphase plate (Figure 4). In cells treated with different concentrations of TN16 most of the mitotic spindles were disheveled. At 50 nM aberrant bipolar and multipolar spindles (70% of total mitotic spindle) were seen. Though chromosomes were aligned as a mid-pole plate, they were slightly puffed up in appearance (Figure 4). But at higher concentrations, i.e. 100 and 200 nM 65% and 75% of total mitotic spindle were multipolar. Chromosomes were improperly aligned. They had puffy ball shaped appearance throughout the spindle, a departure from the usual metaphase plate arrangement (Figure 4). With 200 nM exposure, many aberrant spindles were seen. Some were bipolar with one pole much bigger or smaller than usual. Others were multipolar. Chromosomes were puff shaped ball like in appearance present all over the spindle (Figure 3). In the presence of 200 nM (4 x IC_{50}) TN16, microtubules were completely depolymerized in some of the cells.

TN16 induced apoptotic cell death in HeLa cells

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TN16 induced HeLa cell death. The control cells (vehicle treated) showed no cell death (Figure 5).

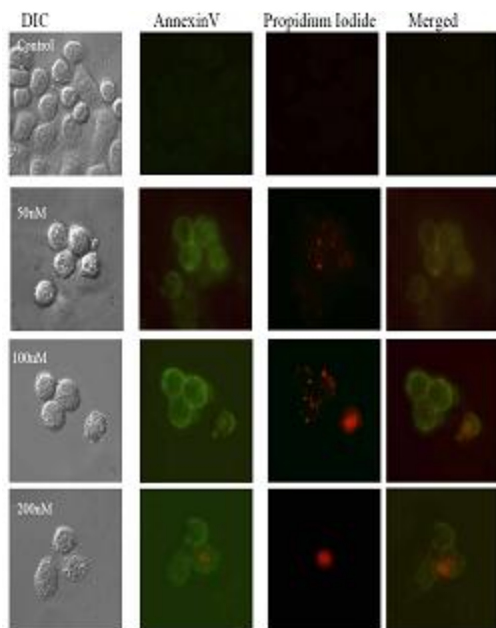

Figure.5: Induction of apoptosis in HeLa cells by TN16

HeLa cells were grown with indicated concentrations of TN16 for 24 h. Cell membrane (green) was stained with Annexin V- FITC and, chromosomes (red) with Propidium Iodide.

Early apoptotic cell death marked by faint red stained nucleus and greenish cell membrane were seen with 50 nM TN16 (Figure 5); whereas late apoptotic cells with dark green membrane and red nuclear spots were observed with 100 & 200 nM (Figure 5). Several red spots in the nuclear region signified chromosome disintegration, a hallmark of late apoptosis. This experiment was repeated three times.

4. Discussion

In this study, we found that TN16 inhibited HeLa cell proliferation by depolymerizing the cellular microtubules. HeLa cell proliferation was blocked in mitosis. The present study is consistent with earlier reports of mitosis blocking effects of TN16 [24]-[26].

By this study, we concluded that TN16 inhibited growth of HeLa cells at IC_{50} of 48±2 nM. The low IC_{50} in nanomolar range is interesting from the point of view of cancer treatment because two major anticancer drugs vinblastine and paclitaxel inhibit Hela cell proliferation in nanomolar concentrations [8].

TN16 caused more than 30% mitotic cells to be present at and above 100 nM (2 x IC_{50}). But the mitotic spindles exhibited abnormal morphology beyond 50nM TN16 exposure. TN16 depolymerized mitotic microtubules strongly giving rise to aberrant spindle formation at and above 50 nM concentration. Multipolar spindles with puffed-up chromosomes were seen. Chromosome congression during mitosis was impaired. Interphase microtubules were also depolymerized at higher concentration of 100 nM (2 x IC_{50}) and 200 nM (4 x IC_{50}). Several other microtubule targeting drugs like paclitaxel, vinblastine, griseofulvin, benomyl, sulfonamides inhibit mitosis by perturbing microtubule structure and dynamics and exhibit similar effects on mitotic microtubule organization [6]-[7], [27].

Microtubules are thought to play important role in cytoskeletal reorganization when a cell is dying by apoptosis [23], [28]. HeLa cells dying through apoptosis in response to different concentrations of TN16 were visualized. Apoptosis was detected by externalization of phosphatidylserine of inner leaflet of the cell membrane which bound Annexin V. FITC labeled Annexin V bound to cell membrane fluoresced green; whereas propidium iodide (PI) entered the necrotic cells and stained the nucleus red. Viable cells were negative for both PI and annexinV; apoptotic cells were positive for annexin V and negative for PI, whereas late apoptotic dead cells displayed both high annexin V and PI labeling. Non-viable cells which underwent necrosis were positive for PI and negative for annexin V. With higher concentration (200 nM) late apoptotic cells were observed, whereas early apoptotic cells were observed with lower concentration (50 & 100 nM). Earlier, microtubule targeting agents like colchicine, nocodazole, etoposide, taxol etc have been shown to induce apoptosis in malignant cell lines [29]-[30]. Together, these data suggest the potential use of TN16 as anticancer compound and warrant further *in vivo* study in future.

5. Conclusions

TN16 exhibits considerable promise as antimitotic agent as evident from works on HeLa cell lines. TN16 inhibited growth of HeLa cells at IC_{50} of 48±2 nM. TN16 depolymerized mitotic microtubules strongly giving rise to aberrant multipolar spindle formation at and above 50 nM concentration.

6. Acknowledgement

The author is thankfully acknowledged to Prof. B. Bhattacharyya, Bose Institute, for guidance, Dr. Dulal Panda, IIT, Mumbai, for using his instrument and laboratory, Dr. Suvroma Gupta, Haldia Institute of technology, for manuscript preparation and CSIR, India for financial support.

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Author Profile

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