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Design and synthesis of triazolyl-naphthyl derivative of β -amyryn and its *in vitro* anti-cancer and apoptotic activities in human nasopharyngeal carcinoma (HK-1) cell line

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Abstract

The purpose of this research work was to design and synthesize triazolyl-naphthyl derivative of β -amyryn (TNB) using click chemistry approach. The cytotoxic activity of TNB was evaluated against HK-1 human nasopharyngeal carcinoma cells using MTT cell viability assay. Fluorescence microscopy indicated cellular morphological changes induced by TNB. Flow cytometry was involved to demonstrate the effect of this compound on cell cycle and apoptosis. The results revealed that TNB inhibited *in vitro* cancer cell growth in dose- and time-dependent manner. The IC₅₀ values of TNB at 24 and 48 hours time intervals were found to be 47.2 and 32.5 μ M respectively. TNB-treated cells exhibited significant dense staining fragmentation called apoptotic bodies, which inferred an early apoptotic event. DNA ladder was more apparent with the increasing the TNB dose. However, no DNA fragments were observed in the control groups. TNB also induced sub-G1 cell cycle arrest and increased fraction of HK-1 apoptotic cells.

Introduction

Nasopharyngeal carcinoma is fairly uncommon in maximum part of the world. Approximately 7 in every 1 million people in America were diagnosed in 2012 (American Cancer Society). Nevertheless, it is more common in parts of Asia, predominantly in the Southern China. Nasopharyngeal carcinoma occurs in children and adults but is most common in males. In comparison to other types of cancers, nasopharyngeal carcinoma is considered very rare but it has been affecting certain ethnic groups in China for decades. In addition to ethnic background, it has been described that nasopharyngeal carcinoma also originates due to dietary habits including high alcohol consumption and salted food (Ning et al., 1990).

Nasopharyngeal carcinoma can be treated by surgery, chemotherapy, radiotherapy or their combination. Even though there are conventional therapies, the serious side effects and toxicities are intolerable to patients (Suárez et al., 2010).

Several compounds isolated from plants have displayed anticancer effect against nasopharyngeal carcinoma (Lui et al., 2009; Lin et al., 2010; Kuo et al., 2012). Conventional anticancer drugs, paclitaxel and cisplatin have been used which worked synergistically with cetuximab to enhance the antitumor effect in nasopharyngeal carcinoma which down-regulates the over-expression of epidermal growth factor receptor (EGFR) (Sung et al., 2005). The aim of the present study was to evaluate the anti-cancer and apoptotic effects of



the β -amyrin and its triazolyl-naphthyl derivative in HK-1 nasopharyngeal carcinoma cells along with evaluating further the mode of action by studying its effect on cellular morphology, cell cycle arrest and DNA fragmentation.

Materials and Methods

Chemicals and reagents

β -Amyrin ($\geq 98\%$ purity) was purchased from Sigma Chemical Company (USA). Growth medium (MEM/RPMI), fetal calf serum, trypsin, penicillin, streptomycin, DMSO, RNase, RIPA Buffer, SDS, MTT dye, propidium iodide, Hoechst 33258 were obtained from Hangzhou Sijiqing Biological Products Co. Ltd, China. All other chemicals and solvents used were of the highest purity grade.

Chemical synthesis of triazolyl-naphthyl derivative of β -amyrin (TNB) using click chemistry approach

To a solution of β -amyrin (**1**, 500 mg) in THF was added cesium carbonate (600 mg) and propargyl bromide (675 mg) and the reaction mixture was stirred at room temperature for 3 hours. The crude product isolated was further subjected to column chromatography to give pure compound (propargylated derivative of β -amyrin) (**2**, 300 mg). To a solution of compound (**2**, 300 mg) in tBuOH-H₂O (2:1, 10 mL) was added sodium ascorbate (3.5 mg) and CuSO₄ (1.5 mg). To this mixture, naphthyl azide (2 equivalents) was added and the reaction mixture was sonicated at 45°C till its completion

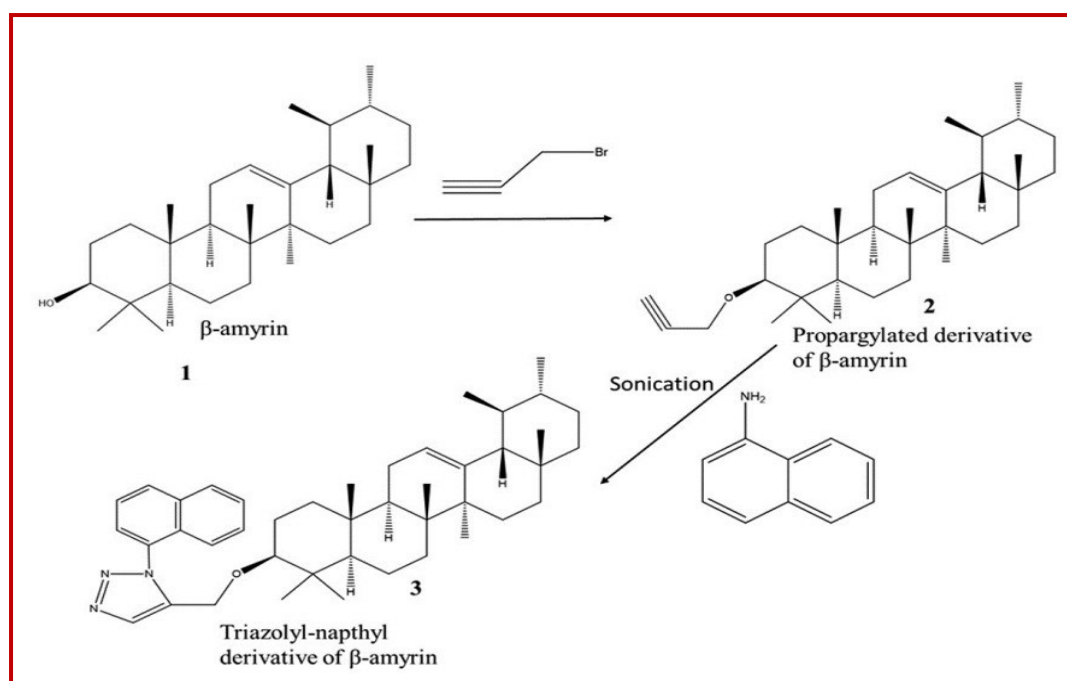
was monitored by TLC. After completion, the reaction mixture was diluted with water and extracted with ethyl acetate. The organic layer was dried over sodium sulfate and purified by column chromatography to give pure compound (**3**) which is the triazolyl-naphthyl derivative of β -amyrin (Scheme 1).

Cell lines, growth medium and treatment conditions

Human nasopharyngeal carcinoma (HK-1) cell line was procured from Shanghai Institute of Cell Resource Center of Life Science (China). Cells were grown in Minimum Essential Medium (MEM) and RPMI supplemented with 10% FCS and 1% penicillin. Cells were cultured in CO₂ incubator (New Brunswick, Galaxy 170R, eppendroff) with an internal atmosphere of 95% air and 5% CO₂ gas and the cell lines were maintained at 37°C. The media was stored at low temperature (2-8°C) and the medium for cryopreservation contained 10% FCS and 5% DMSO in growth medium.

Cell viability testing by MTT assay

The cytotoxic effects of TNB on the cell viability of HK-1 human nasopharyngeal carcinoma cells was evaluated by MTT assay. HK-1 cell line was seeded in a 96-well plate at 3×10^3 cells per well and then treated with 0, 3, 6, 12, 24, 50 and 100 μ M dose of TNB for 24 and 48 hours. The cell plates were then treated with MTT solution (10 μ L) in PBS for two hours. The formazan crystals then formed were dissolved in DMSO and the absorbance was measured on a microplate reader (FLUOstar Optima, BMG Labtech, Germany). Data were expressed as a percentage of control measured in



Scheme 1: Chemical synthesis of triazolyl-naphthyl derivative of β -amyrin using click chemistry approach

the absence of TNB compound.

Fluorescence microscopic study

HK-1 cells were seeded on a chamber slide (Thermo Scientific Nunc Lab Tek II) at a cell density of 3×10^5 cells per chamber. The cells were treated with 0, 12, 48 and 100 μM TNB for 48 hours. Afterward, 20 $\mu\text{g}/\text{mL}$ of acridine orange and 20 $\mu\text{g}/\text{mL}$ of propidium iodide were added to each chamber. It was then observed under Fluoview 1000 laser scanning confocal microscope (Olympus IX 81 motorized inverted microscope).

DNA fragmentation analysis after TNB treatment

The HK-1 cells (3×10^5 cells/dish) were plated in 6-cm dish and then subjected to the treatment of various concentrations (0, 12, 48 and 100 μM) of the TNB for 48 hours. After drug treatment, the cells were washed with ice-cold PBS and resuspended in lysis buffer (25 mM Tris-HCl, pH 7.4, 5 mM EDTA and 0.6% SDS) with 1.0 mg/mL RNase A for 20 min at 50°C. Then proteinase K was added and the cells were incubated overnight. Separation of DNA was done using 2% agarose gel and detected under UV light after staining with ethidium bromide (ETBR).

Effect of TNB on cell cycle phase distribution

Cell cycle was analyzed by FACSC alibur instrument (BD Biosciences, USA) equipped with Cell Quest 3.3 software. Briefly, HK-1 cells (3×10^5) cells were treated with various concentrations of TNB (0, 12, 48 and 100 μM) for 48 hours. After this, the cells were collected, washed with ice-cold PBS buffer, fixed with 70% alcohol at 4°C for 12 hours, and stained with propidium iodide in the presence of 1% RNAase A at 37°C for 30 min before analysis by flow cytometry.

Annexin V binding assay/Quantification of apoptotic cell death

To establish and confirm cells undergoing apoptosis, we performed annexin V binding assay through flow cytometry. Briefly, HK-1 cancer cells were treated with the TNB (0, 12, 48 and 100 μM) for 48 hours, and then treated and untreated cells were harvested by trypsinization. Harvested cells were then incubated in annexin V-FITC (25 ng/mL) and propidium iodide (25 $\mu\text{g}/\text{mL}$), at room temperature for 30 min in the dark, and analyzed using a FACS Calibur flow cytometer (BD Bioscience) taking a minimum 10,000 cells in each sample.

Statistical analysis

All data were derived from at least three independent experiments. The results were expressed as the mean \pm SD. Differences between groups were analyzed using the Student's t-test. $p < 0.05$ was considered statistically significant.

Results

***In vitro* cytotoxic effect of TNB**

We evaluated *in vitro* inhibitory effect of TNB using the MTT viability assay against HK-1 cells at indicated concentrations of 0, 3, 6, 12, 24, 50 and 100 μM for 24 and 48 hours. The results revealed that TNB had potent cytotoxic effects on HK-1 nasopharyngeal cancer cells. It revealed both concentration-dependent as well as time-dependent growth inhibitory effects against these cells (Figure 1). The efficacy of the compound was evaluated at two different time intervals by calculating the IC_{50} values, which is the concentration that causes 50% growth inhibition. The IC_{50} values of TNB at 24 and 48 hours time intervals were found to be 47.2 and 32.5 μM respectively.

Fluorescence microscopic study of TNB-induced cellular morphological changes

In the case of fluorescence microscopy, HK-1 cells were stained and evaluated for nuclear shape using a fluorescence microscope with Hoechst 33258 staining (Figure 2A-D). The results revealed that TNB-treated cells showed considerable chromatin condensation or dense staining fragmentation called apoptotic bodies, which inferred an early apoptotic event. The presence of such apoptotic bodies was related to the TNB dose. HK-1 cells underwent the morphologic changes typical of apoptosis after treatment with the compound. Uniformly spherical HK-1 cells with normal morphology were observed in untreated cultures, whereas chromatin condensation, chromosomal DNA cleavage were observed when HK-1 cells were treated with TNB at different concentrations.

Effect of TNB on DNA fragmentation

Besides the morphological changes of apoptosis in TNB-treated HK-1 cells, DNA fragmentation was also studied by evaluation of the formation of DNA ladder. As shown in Figure 3, DNA ladder appeared to be more evident with the increasing TNB concentration, however, no DNA fragments were observed in the control groups (Figure 3; 0 μM). However, 12, 48 and 100 μM doses of the compound after 48 hours exposure led to a considerable upsurge in DNA fragmentation (Figure 3; right panel). The DNA fragmentation is a symbol of apoptosis, further confirming that the TNB compound induced cell death through apoptosis.

Effect of TNB on cell cycle phase distribution

Apoptosis and cell cycle are strictly related biochemical processes, and any interference in cell cycle progression may eventually lead to apoptotic cell death. DNA content in cells was detected by propidium iodide staining and flow cytometry. In order to elucidate the mechanistic pathway of the growth inhibitory effect

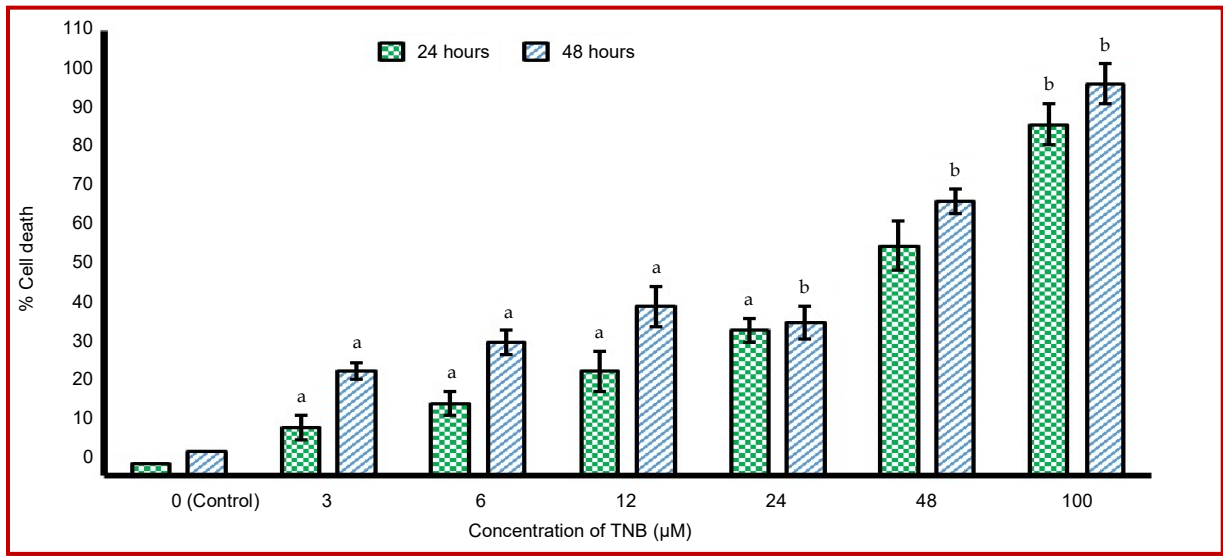


Figure 1: Cytotoxic effect of triazolyl-naphthyl derivative of β -amyrin (TNB) in human nasopharyngeal carcinoma cells (HK-1). Data are shown as the mean \pm SD of three independent experiments. ^a $p < 0.05$, ^b $p < 0.01$, vs 0 μ M (control)

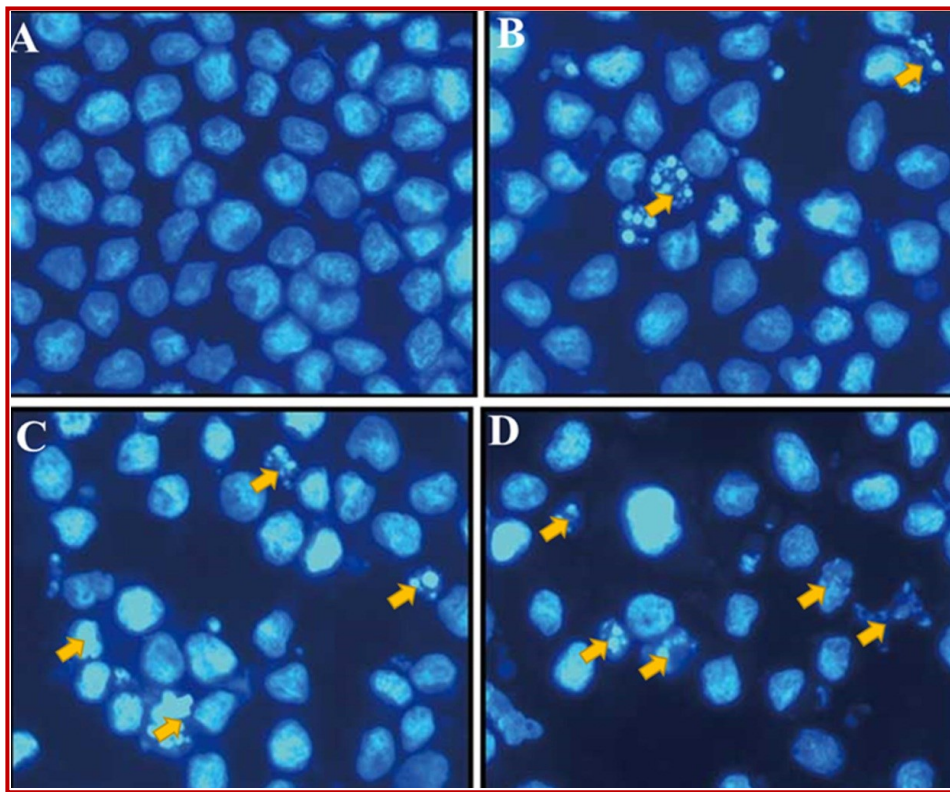


Figure 2: Effect of TNB on the nuclear morphology of HK-1 cancer cells using Hoechst 33258 staining at actual magnification 200x. HK-1 cells were treated without (A) and with TNB 12 μ M (B), 48 μ M (C), and 100 μ M (D) for 48 hours. Colored arrows represent apoptotic cells exhibiting chromatin condensation and nuclear fragmentation

exerted by TNB in HK-1 cancer cells, flow cytometry analysis was carried out to ascertain whether TNB induces cell cycle arrest in this cell line. The results revealed that TNB induces sub-G1 cell cycle arrest and increases the fraction of apoptotic cells in HK-1 cells in a dose-dependent manner (Figure 4). At the same time,

the fraction of G0/G1 cells decreased with the increase in the dose of TNB. The percentage of cells in S and G2/M phase more or less remained unaffected.

TNB induced both early and late apoptosis

Annexin V/PI double staining was used to detect

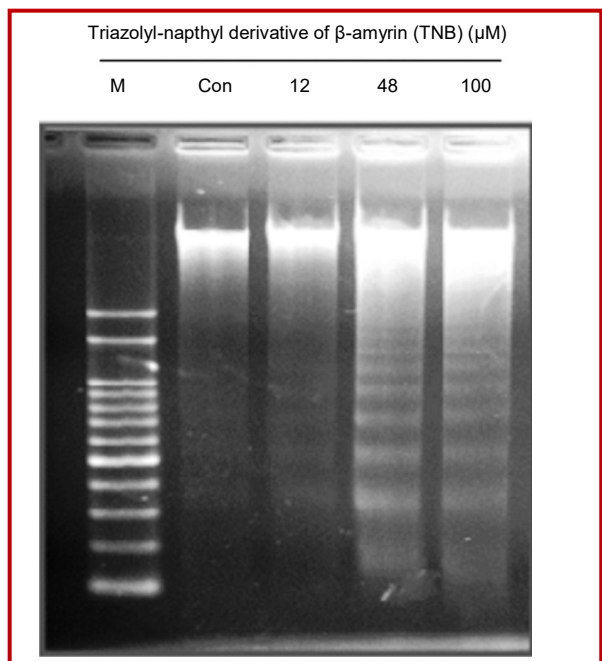


Figure 3: TNB induces DNA fragmentation in human nasopharyngeal cancer cells (HK-1). The cells were treated with 0, 12, 48 and 100 μM of the compound for 48 hours. Cells from each sample were harvested for DNA gel electrophoresis as described in Materials and Methods

apoptosis in the human nasopharyngeal cancer cells (HK-1) (Figure 5A-D). HK-1 cells were treated with different concentration (0, 12, 48 and 100 μM) of TNB for 48 hours. TNB induced both early and late apoptosis in a concentration-dependent manner (Figure 5B-D) as compared to the untreated control cells (Figure 5A). The different quadrants Q1, Q2, Q3 and Q4 represent

necrotic cells, late apoptotic cells, viable cells and early apoptotic cell population respectively. Percentage of apoptotic cells increases from 5.6% in control cells (A), to 29.3%, 37.1% and 45.2 % in 12 μM (B), 48 μM (C) and 100 μM (D) TNB-treated cells respectively.

Discussion

In the present study, it was reported that triazolyl-naphthyl derivative of β -amyirin (TNB) exhibited potent antitumor activity in human nasopharyngeal cancer cells (HK-1) by inducing early and late apoptosis, cell cycle arrest in sub-G1 phase and DNA fragmentation. Cell viability assay using MTT showed that TNB inhibited *in vitro* cancer cell growth in a dose-dependent and time-dependent manner. The IC_{50} values of TNB at 24 and 48 hours time intervals were found to be 47.2 and 32.5 μM respectively. Fluorescence microscopy studies revealed that TNB-treated cells exhibited significant chromatin condensation or dense staining fragmentation called apoptotic bodies, which inferred an early apoptotic event. Homogenously spherical HK-1 cells with normal morphology were seen in untreated cells, whereas chromatin condensation and chromosomal DNA cleavage were observed when HK-1 cells were treated with TNB at different concentrations. Further, gel electrophoresis indicated that DNA ladder seemed to be more apparent with the increasing the TNB dose, however, no DNA fragments were observed in the control groups. 12, 48 and 100 μM doses of the compound after 48 hours exposure led to a considerable upsurge in DNA fragmentation. Flow cytometry studies indicated that TNB induces sub-G1 cell cycle arrest and increases the

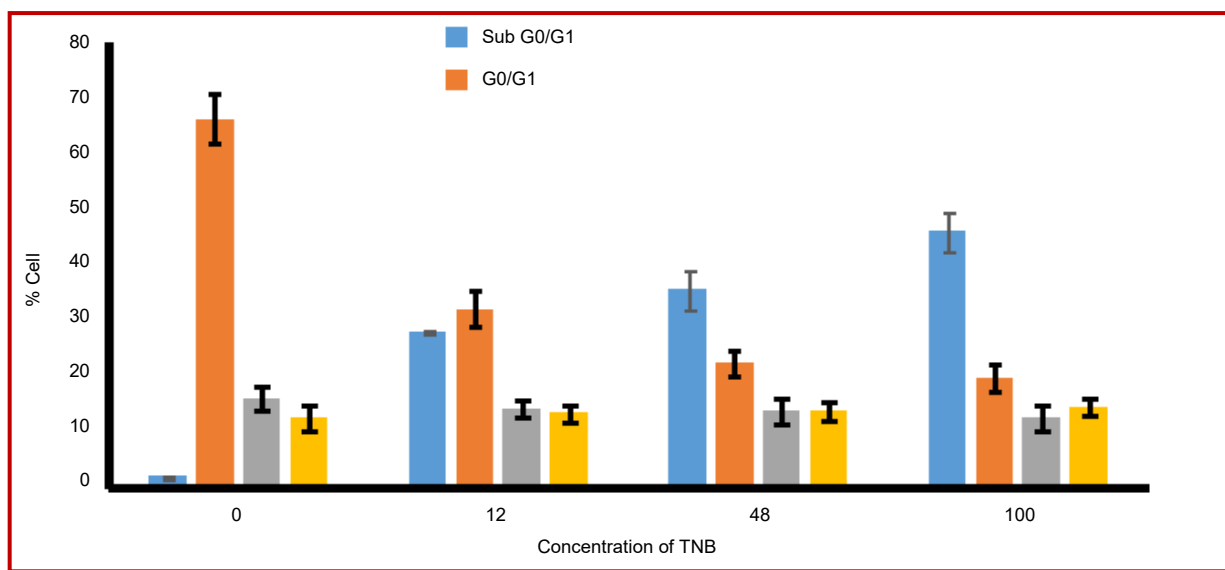


Figure 4: Effect of TNB on the cell cycle analysis of the HK-1 nasopharyngeal carcinoma cells. The cells were treated with 0, 12, 48 and 100 μM of the compound for 48 hours and then analyzed by flow cytometry. Note the percentage of sub-G0/G1 (apoptotic cells) cells increases with increase in dose of TNB

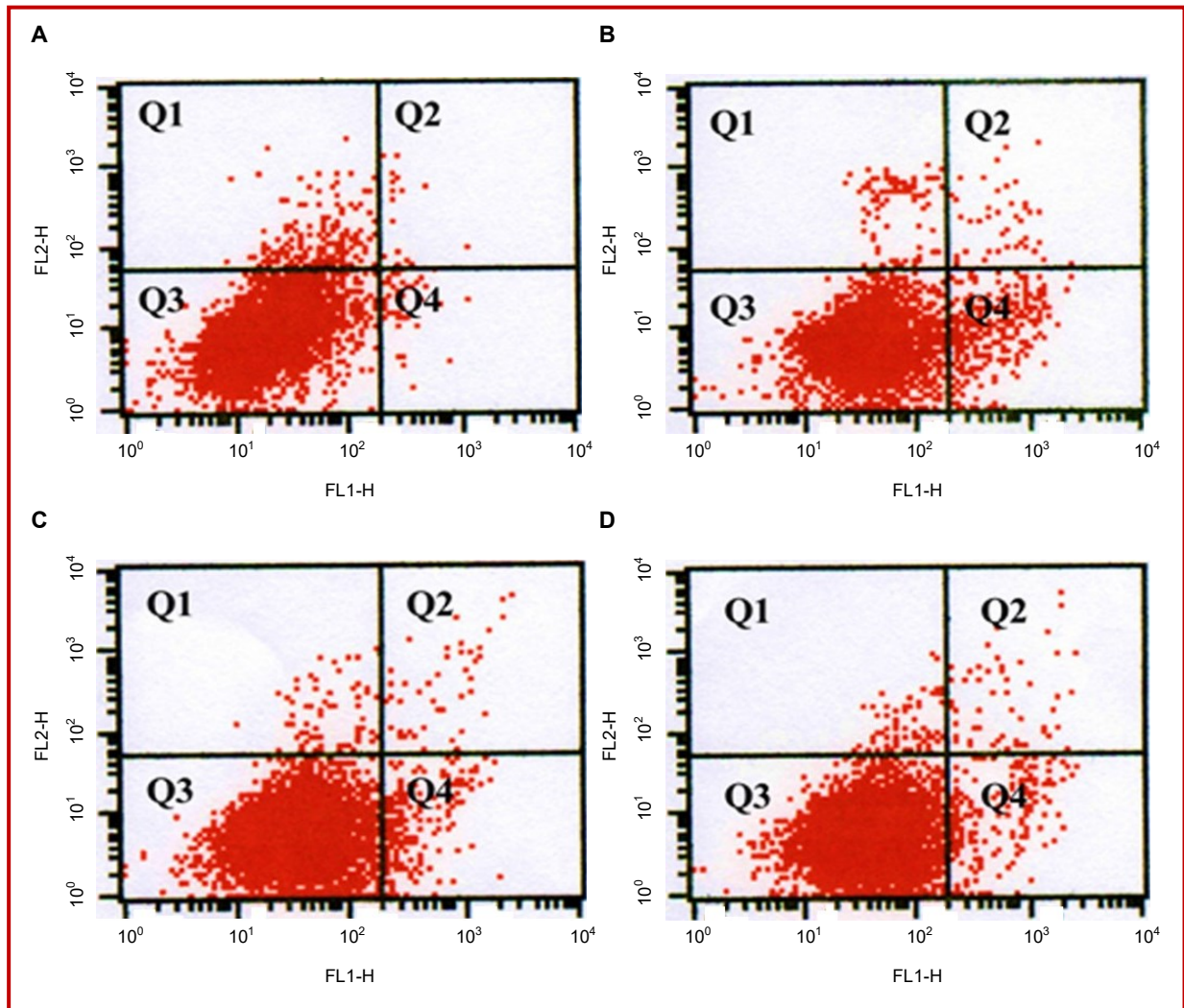


Figure 5: Quantification of TNB-induced apoptosis in human nasopharyngeal cancer cells (HK-1). The cells were subjected to different doses of TNB (0, 12, 48 and 100 μ M) for 48 hours and analyzed by flow cytometry with annexin V-FITC/PI staining. The different quadrants Q1, Q2, Q3 and Q4 represent necrotic cells, late apoptotic cells, viable cells and early apoptotic cell population respectively. Percentage of apoptotic cells increases from 5.6% in control cells (A), to 29.3%, 37.1% and 45.2% in 12 μ M (B), 48 μ M (C) and 100 μ M (D) TNB-treated cells respectively

fraction of HK-1 apoptotic cells in a dose-dependent manner. However, the percentage of G0/G1 cells diminished with the increase in the dose of TNB. TNB induced both early and late apoptosis in a concentration-dependent manner as compared to the untreated control cells.

β -amyirin is a pentacyclic triterpenoid synthesized by a number of plants. This compound is often found in plant extracts along with its isomer α -amyirin differing in the placement of one methylene group (C-19 and C-20 respectively). Literature survey revealed that no such research work has been reported on triazolyl-naphthyl derivative of β -amyirin (TNB) so far. However, ester derivatives of amyirin have been reported to induce apoptotic cell death in HL-60 leukemia cells. This study also reported the effect of these ester derivatives on

cellular morphology, DNA fragmentation, cell cycle arrest, externalization of phosphatidylserine and activation of caspases 3/7 (Barros et al., 2011). It has also been reported that α - and β -amyirin isomers protect the liver against acetaminophen-induced injuries and ameliorates L-arginine-induced acute pancreatitis. α -Amyirin esters have also been reported to show substantial anti-inflammatory activity (Oliveira et al., 2005; Melo et al., 2010; Akihisa et al., 2010). However, our study focused on triazolyl derivatives of beta-amyirin rather than alpha-amyirin. Further, our study focused on a different cancer cell line namely nasopharyngeal carcinoma cell line. In literature we could not find reports on the anti-cancer activity of either alpha-amyirin or beta-amyirin or their derivatives against nasopharyngeal cancer cells and as such this report is the first such report on this cell line.

Conclusion

TNB synthesized by using click chemistry approach, inhibit nasopharyngeal cancer cell (HK-1 cell line) growth by inducing apoptosis, chromatin condensation, DNA fragmentation and sub-G1 cell cycle arrest.

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Conflict of Interest

Authors declare no conflict of interest to reveal

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