Effects of Navelbineon Cell Proliferation of Hela Cell Culture

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Abstract

In this study, antiproliferative effects of navelbine belonging to vinca alkaloids family and used as an anti-microtubule agents was evaluated on HeLa cell line, originating from human cervix carcinoma. For this purpose, cell kinetic parameters including cell viability analysis, mitotic index, labelling index and apoptotic index were used. The results showed that while there was a significant decrease in rates of viability and labelling index values, there was a significant increase in mitotic index values regarding the cell cycle effects depending on G_2/M block for all experimental groups. Also there was a significant increase in apoptotic index values depending on interference with the dynamics of microtubules. Differences between control and all experimental groups were statistically significant ($p<0.01$) for all applications.

Key words: Cervix Cancer, Navelbine, Cell Viability, Mitotic Index, Labelling Index, Apoptotic Index

Introduction

Microtubules are major components of the cytoskeleton. They have an important role in many cellular events, and they are also vital in cell division. Therefore, microtubules attract a lot of attention in designing anticancer drugs. Tubulin-binding agents are commonly used in the treatment of cancer as chemotherapeutic drugs and these agents are also named as anti-microtubule or microtubule-targeted agents (Pasquier and Kavallaris, 2007). Their bioactivity is dependent on altering microtubule dynamics, and this alteration disrupts formation of mitotic spindles (Klotz et al., 2012). Vinca alkaloids have been widely used for a long time traditionally in the treatment of solid tumors and also leukemias; and they are members of antimicrotubule agents (Jordan et al., 1991). Three kinds of vinca alkaloids, i.e. vincristine, vinblastine and vinorelbine (VLB), are among the most widely used classes of antineoplastic agents. These alkaloids have cytotoxic effect on cancer cells; they inhibit the dynamics and assembly behavior of microtubule during metaphase and thus, cell growth stops at this stage (Li et al., 2012). Navelbine is produced semi-synthetically from the precursor alkaloids and represents the latest clinically approved vinca alkaloid (Johnson et al., 1996). Navelbineis considered to be a mitotic spindle poison, i.e. it impairs chromosomal segregation during mitosis by distrupting the mitotic spindle. It blocks cells at G2/M when administered at concentrations close to IC_{50}: at higher concentrations polyploidy occurs. Microtubules derived from tubulin polymers and they are considered to be the principal target of navelbine (Gregory and Smith, 2000). Vinca alkaloids bind both to tubulin and microtubules, and their activities are dependent on the administered concentration to a high extent. When administered at relatively high concentrations, they depolymerize microtubules, dissolve spindle microtubules and arrest cells at mitosis; and when administered at higher concentrations (mM), they induce the aggregation of tubulin into paracrystalline arrays. On the contrary, when administered low concentrations, vinca alkaloids are known to suppress microtubule dynamics; they demonstrate this effect without depolymerizing spindle microtubules, but mitosis is still arrested and apoptosis is induced(Zhou and Giannakakou, 2005).

In the present study, it is aimed to investigate the antiproliferative effects of navelbine, which is a microtubule targeted agent. For this purpose, HeLa cell line was used as cervix carcinoma model. Therefore cell kinetic parameters including cell viability analysis, mitotic index and labelling index and apoptotic index were evaluated.

Methods

Cell culture

The HeLa cell line used in this study was obtained from European Cell Culture Collection (CCL) and were cultured in Medium-199 (M-199, Sigma, USA) containing 10% fetal bovine serum (FBS, Gibco Lab), 100 µg/ml streptomycin (Streptomycin sulphate, I. E. Ulugay), 100 IU/ml penicilin (Pronapen, Pfizer), amphotericin B (Sigma, USA) and 2 mM glutamine at 37^oC in humidified atmosphere of 5% CO_2. $NaHCO_3$ was used to adjust the pH of the medium to 7.4.

Drug doses

In order to decide on the navelbine concentrations to be used in our study, we performed a literature research and on previous *in vitro* and clinical studies. First, 10 µM stock solution was prepared with M-199 which was supplemented with FBS at a ratio of 10%. Stock solution was diluted to obtain three different doses and these doses were determined as dose 1 (D_1) = 0.1 µM, dose 2 (D_2) = 0.5 µM and dose 3 (D_3) = 1.0 µM, respectively. HeLa cell cultures were exposed to these 3 doses for a period of 12, 24, 48 and 72 h.

Cell viability analysis

After being exposed to navelbine, cell viabilities were analyzed with MTT (Thiazolyl Blue Tetrazolium Bromide, Sigma). MTT is known to effect mitochondrial dehydrogenase enzyme of the cells and reduces tetrazolium salts to colored formazan compounds. HeLa cells were seeded into 96 well plates at density of $2x10^4$ cell per well. At the end of the experimental period, medium in each well was removed and 40 μl fresh MTT solution (5 mg/ml in PBS) was added into each well and cells were incubated at 37^{o}C for 4 h. Then, DMSO (Dimethyl Sulfoxide, Sigma) was added into each well and cells were shaken thoroughly for 1 h on a shaker. After this, absorbances of the samples were measured against a background control as a blank using an Elisa reader (µQuant, Bio-Tek Instruments Inc) at 450-690 nm.

Determination of optimal dose with cell viability analysis

For the determination of the most effective dose of navelbine for HeLa cells, three different doses (D_1= 0.1 µM, D_2= 0.5 µM, D_3= 1.0 µM) were applied to cell culture for 24 h. Cytotoxic effects of the doses were evaluated with MTT assay. At the end of this process, the most effective dose determined according to absorbance values of different drug concentrations was used for the following parameters:

Mitotic index analysis

MI was studied using the Feulgen staining method. This technique enables us to identify chromosomal material or DNA in cell specimens. It depends on acid hydrolysis of DNA, therefore fixating agents using strong acids must be avoided (Bedi and Goldstein, 1976; Kjellstrand, 1980).Before staining the cells with Feulgen, they were treated with 1 N HCl at room temperature for 1 min, and then hydrolized with 1 N HCl for 10.5 min at 60°C. After staining of the slides with the reagent, slides were rinsed for a couple of minutes in distilled water, and then stained with 10% Giemsa stain solution at pH 6.8 for 3 min and washed twice in phosphate buffer. After staining, slides were rinsed in distilled water and were air-dried. Finally, MI was calculated by counting metaphases, anaphases and telophases for each control and experimental groups. At least 3,000 cells were examined using light microscope from each slide to determine MI.

Preparing ^{3}H-thymidine

Stock solution was prepared by adding 9 ml deionized water to a vial containing 1 mCi/ml ^{3}H-thymidine (TRA-120, Amersham, England). Then 1 mCi/ml solution was diluted to 1 µCi/ml with cell culture medium. The cells were labelled with this solution.

Application of Drug and ^{3}H-Thymidine

Drug concentrations administered to HeLa cells were determined according to previous *in vitro* and clinical studies. Cells were exposed to optimum dose of navelbine for 0-72 h for the determination of all kinetic parameters of the cell. At the end of this periods, cells were incubated in medium containing 1 µCi/ml ^{3}H-thymidine for 20 min. to determine the labeling index parameter, and then cells were labelled and fixation was carried out.

Labelling index (LI)

Giemsa stain was used to stain the autoradiograms at 16^{o}C for 3 min. For each drug concentration and time period of each application, 3000 cells were counted under light microscope and percentage of cell labelling was calculated.

Autoradiography

After labelling, the cells were fixed with Carnoys fixative [consisting of ethanol: glacial acetic acid (3: 1)] and 2% perchloric acid was used to wash away the remaining radioactive materials at 4^{o}C for 30 minsand this removal process was applied twice. After preparing the slides, they were coated with K.2 gel emulsion (Ilford, England) prepared with distilled water at 40^{o}C for the determination of thymidine labelling index. After 3 days of exposure at 4^{o}C, autoradiograms were washed with D- 19 b developer (Kodak) and fixed with Fixaj B (Kodak).

Labelling index (LI)

Giemsa stain was again used to stain autoradiograms were again stained with at 16^{o}C for 3 min., 3000 cells were counted under light microscope for each drug concentration and time period of each application, and percentage of cells that have been labelled was calculated.

Apoptotic Index (AI)

The apoptotic index i.e. the percentage of cells undergoing apoptosis, was studied with the help of a fluorescence microscope. For the determination of AI, cells were fixed with methanol and stained with 4'-6 diamidine-2 phenyl indol (DAPI). Following extensive washing in phosphate-buffered saline (PBS), slides were scored under fluorescence microscope. For evaluation of AI, at least 100 cells were counted for control and each experimental group.

Statistics

Values of proliferation rate, MI and AI were evaluated relative to controls and also relative to each other. One-way ANOVA test was used to analyze the values obtained from all experimental groups. DUNNETT's test was used to reveal the significance between control and experimental groups and Student's t-test was used to determine the significance between experimental groups.

Results

Determination of optimal dose with cell viability analysis

The absorbance values of each dose for 24 hours were $446.387x10^{-3}$ for control, $388.215x10^{-3}$ for D_1, $294.736x10^{-3}$ for D_2 and $219.176x10^{-3}$ for D_3 (Figure 1). The results indicated that 24 hours after administration of Navelbine to HeLa cells, viability values were 87 % for D_1, 66% for D_2 and 49 % for D_3 according to control group which was considered as 100% (Figure 2). The differences between control and all experimental groups were statistically significant ($p<0.01$).

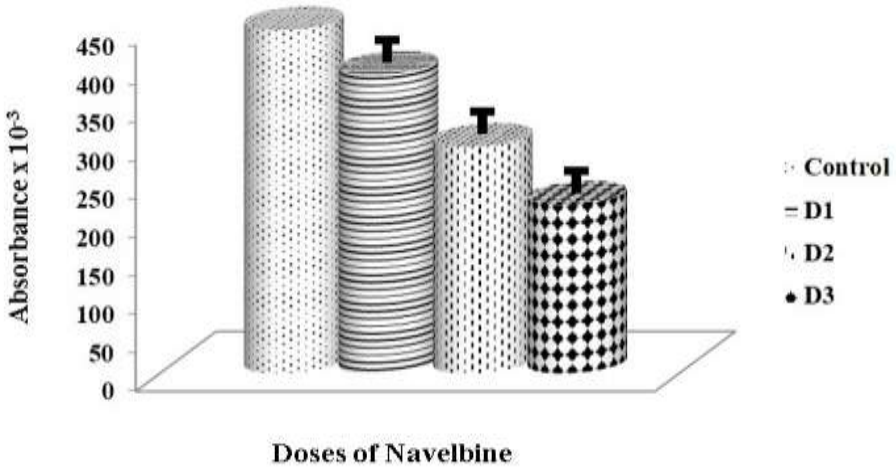

Figure 1: Absorbance values of mitochondrial dehydrogenase enzyme activity of HeLacells treated with three different doses of Navelbine(D_1= 0,1 µM, D_2= 0,5 µM, D_3= 1µM) for 72 h (450-690 nm).

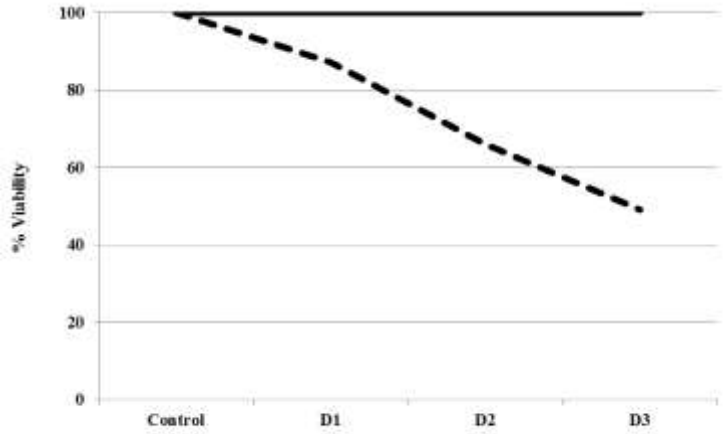

Figure 2: Viability % values of HeLa cells treated with three different doses of Navelbine (D_1= 0,1 µM, D_2= 0,5 µM, D_3= 1µM) for 24 h.

Determination of cytotoxic activity with cell viability analysis

After administration D_3 dose of Navelbine to HeLa cells for 0-72 h, it was observed that there was a significant decrease in cell viability values. While absorbance values of control groups were $248.354x10^{-3}$, $314.213x10^{-3}$, $357.068x10^{-3}$, $396.118x10^{-3}$ and $447.438x10^{-3}$ respectively for 0, 12, 24, 48 and 72 h; absorbance values of experimental groups were $247.712x10^{-3}$, $236.311x10^{-3}$, $232.712x10^{-3}$, $229.654x10^{-3}$ and $218.123x10^{-3}$respectively for 0, 12, 24, 48 and 72 h as shown in Figure 3.After administration D_3 dose of Navelbine to HeLa cells, viability values were 99.741 % for 0 h, 75.207 % for 12 h, 65.173 % for 24 h, 57.976 % for 48 h and 48.749 % for 72 h according to control groups which was considered as 100% (Figure 3). The differences between the control and all experimental groups were significant ($p<0.01$).

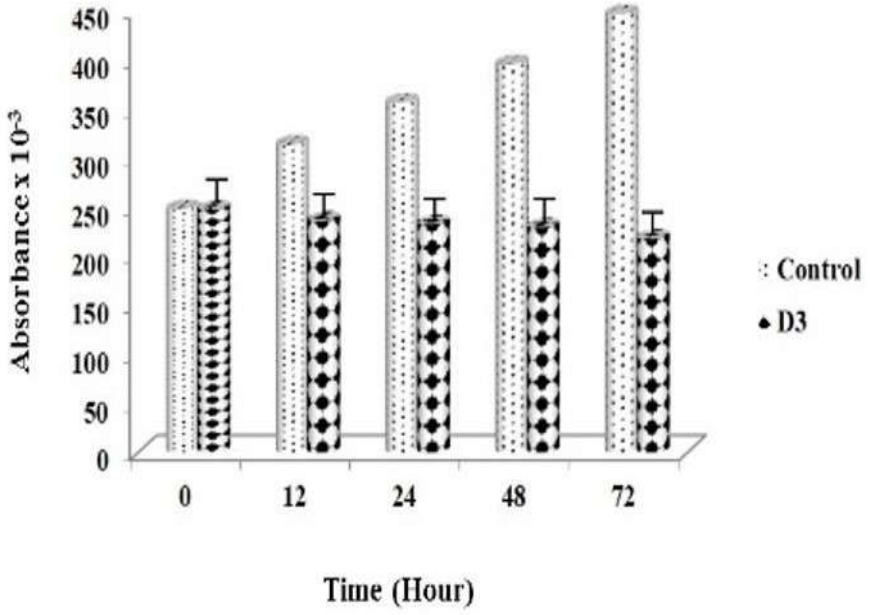

Figure 3: Absorbance values of mitochondrial dehydrogenase enzyme activity of HeLa cells treated with D_3 dose of Navelbine (D_3= 1µM) for 0-72 h (450-690 nm).

Mitotic index (MI)

After administration D_3 dose of Navelbinefor 0-72 h, mitotic index values of HeLa cells were increased significantly.The differences between the control and all experimental groups were significant ($p<0.01$). As seen in the Figure 4, D_3 dose of Navelbine which was the most effective dose among all doses increased mitosis from7.38% to 8.06% at 24 h; from 5.42% to 10.56% at 48 h; from 4.87% to 12.88% at 72 h.

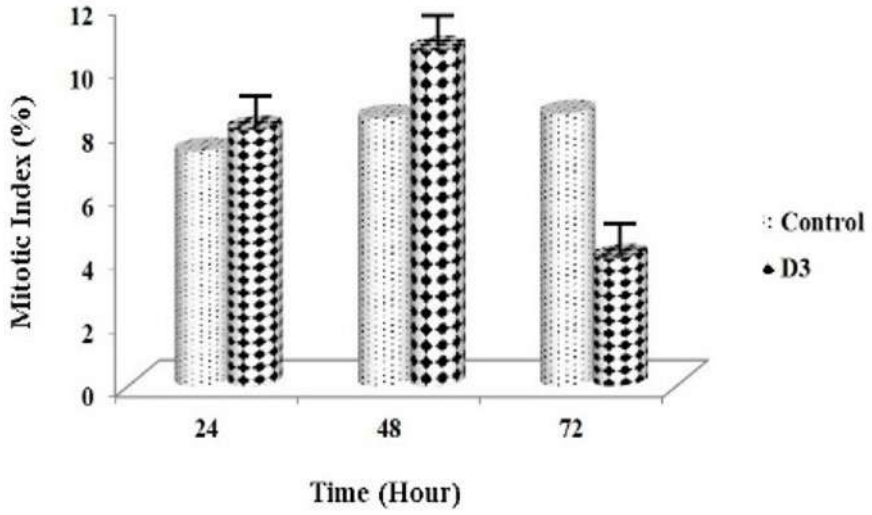

Figure 4: Mitotic index (%) values of HeLa cells treated with D_3 dose of Navelbine (D_3= 1µM) for 0-72 h.

Labelling index (LI)

After administration D_3 dose of Navelbine for 0-72 h,labelling index values of HeLa cells were decreased significantly. The differences between the control and all experimental groups were significant ($p<0.01$). D_3 dose of Navelbine which was the most effective dose among all doses decreased DNA synthesis from 5.16% to 3.46% at 24 h; from 4.88% to 2.21% at 48 h; from 3.32% to 0.11% at 72 h (Figure 5).

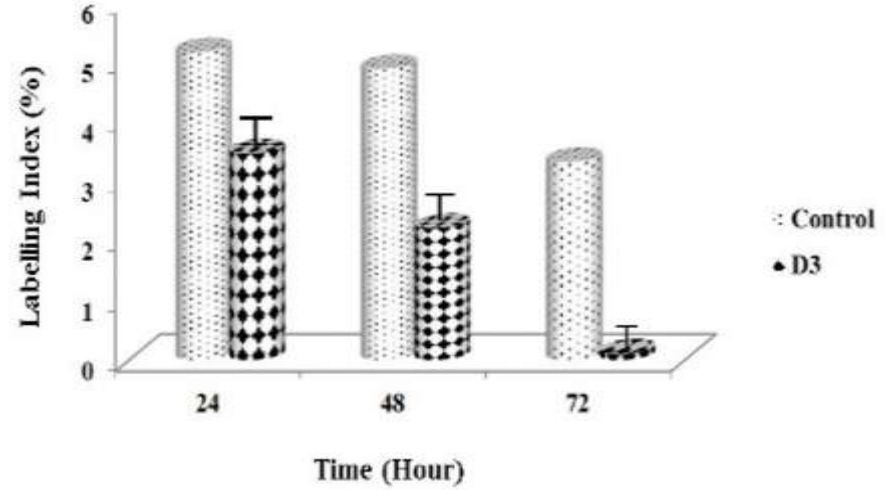

Figure 5: Labellingindex (%) values of HeLacellstreatedwith D_3dose of Navelbine (D_3= 1µM) for 0-72 h.

Apoptotic index

After administration of Navelbineto HeLa cell line for 24, 48 and 72 h, apoptotic index values of the cells were increased significantly. The differences between the control and all

experimental groups were significant ($p<0.01$). As seen in the Figure 6, apoptosis increased from 8.33% to 18.26% at 24 h; from 9.62% to 21.42% at 48 h; from 10.22%, 28.45% at 72 h.

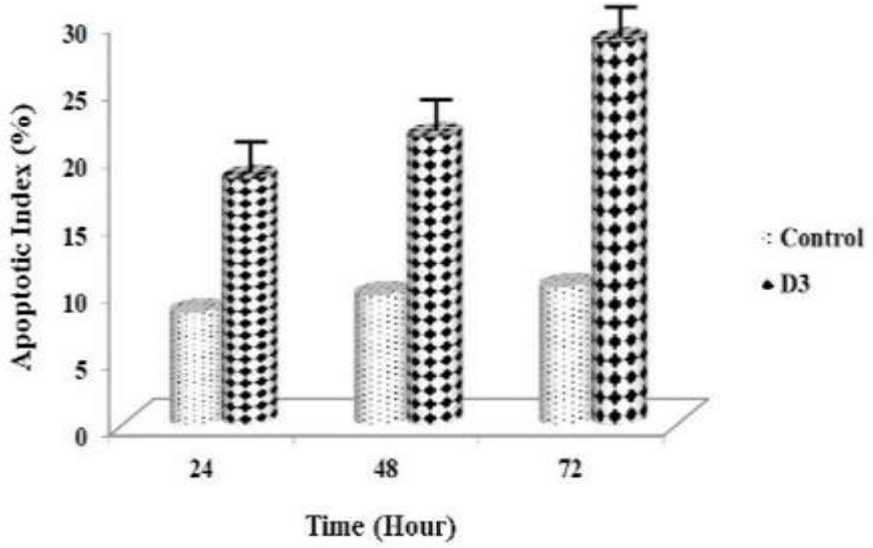

Figure 6: Apoptoticindex (%) values of HeLacellstreatedwith D_3dose of Navelbine (D_3= 1µM) for 0-72 h.

Discussion

Highly dynamic mitotic-spindle microtubules are among the most successful targets for the treatment of cancer. Microtubule-targeted drugs, including paclitaxel and *Vinca* alkaloids were previously considered to show their effects mainly by increasing or decreasing cellular microtubule mass (Jordan and Wilson, 2004). *Vinca* alkaloids (vinblastine and vincristine) have been used successfully in cancer chemotherapy for different tumors types for a long time (Kreis, 1995).*Vinca* alkaloids alter mitotic spindle functions and therefore they prevent cell cycle progression and result in mitotic block (Kruczynski et al., 1998; Jean-Decoster et al., 1999; Ngan et al., 2001; Jordan, 2002). *Vinca* alkaloids induce apoptosis just like other microtubule damaging agents (MDAs). These cells that are exposed to *Vinca* alkaloids die due to apoptosis and during this process they manifest some typical morphological changes along with DNA fragmentation These drugs promote apoptosis in cancer cells (Fan et al., 2000).

In our study, there was a significant increase in both mitotic index values depending on G_2 blockage and apoptotic index values after treatment of HeLa cells with 0.1 µM, 0.5 µM, 1.0 µM concentration of the drug. In a study by Pourroy, neural and non-neural cell lines were treated with low concentrations of another *Vinca* alkaloid, vinflunine, at the nanomolar level. Vinflunine was found to induce G_2/M block and apoptosis in both cell lines. But as for SHEP, SH-SY5Y, and IMR32 cell lines, vinflunine failed to induce G_2/M block, however it induced apoptosis (Pourroy et al., 2004). Inducing G_2/M block and apoptosis for neural and non-neural cell lines except three cell lines support the results of our study.

In mitotic index analysis by Ngan, HeLa cells treated with vinflunine, navelbine, and vinblastine. HeLa cell proliferation for vinflunine, navelbine, and vinblastine were found to be18, 1.25, and 0.45 nM, respectively,; similar to the concentrations that induced mitotic

block at the metaphase/anaphase transition (38, 3.8, and 1.1 nM, respectively), indicating that mitotic block is a major contributor to antiproliferative action for all three drugs. The ratio of the number of cells in anaphase to those in metaphase decreased to zero over the concentration range that induced mitotic block, indicating a block specifically in metaphase. At concentrations of 75 nMvinflunine, 8 nMnavelbine, and 2.2nM vinblastine, no cells were observed to be in anaphase. Thus, the block occurred specifically at the transition from metaphase to anaphase (Ngan et al., 2001).

In previous studies performed with mitotic modulators, it is not surprising to see that G_2/M arrest was induced by anti-microtubule agents; this phenomenon leads to mitochondrial permeability transition and then triggering of the downstream cascades of the intrinsic apoptotic pathway (Chang et al., 2009).

When cells enter mitosis, many microtubule interacting agents may interfere with the dissociation of chromosomes, thus activating cell cycle checkpoints and inducing G_2/M arrest and/or apoptosis. The G_2/M checkpoint is quite important since it provides time for DNA repair, whereas apoptosis may function to remove irreparably damaged cells (Yang et al., 2004).

^{3}H-thymidine labelling index values obtained with in our present study indicates that ^{3}H-thymidine labelling index values of 24-72 h are in concordance with the results of the cell viability values of 0-72 h.

In accordance with the effects of navelbine on cell kinetic parameters, it is considered that this drug, which is already being used will continue to be administered in various types of cancers.

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