

RESEARCH ARTICLE

The Effect of Abraxane on Cell Kinetic Parameters of HeLa Cells

Nurcan Gurses^{1*}, Mehmet Topcul²

Abstract

Abraxane (nab-paclitaxel) is a member of the group of nano chemotherapeutics. It is approved for metastatic breast cancer and non small cell lung cancer. Trials for several cancer types including gynecological cancers, head and neck, and prostatic cancer are being studied. In this study, the antiproliferative and apoptotic effect of abraxane was evaluated on HeLa cell line originated from human cervix carcinoma. Three different doses ($D_1=10$ nM, $D_2=50$ nM, $D_3=100$ nM) were administered to HeLa cells for 24, 48 and 72 h. The 50 nM dose of abraxane decreased DNA synthesis from 4.62-0.08%, mitosis from 3.36-1.89% and increased apoptosis from 10.6-30% at 72 h. Additionally, tripolar metaphase plates were seen in mitosis preparations. In this study, abraxane effected cell kinetic parameters significantly. This results are consistent with other studies in the literature.

Keywords: Abraxane - cervical cancer - HeLa - nanodrugs - nab-paclitaxel - *in vitro* - ³H Thymidine

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Introduction

Paclitaxel (PTX) is a chemotherapeutic agent that has been used to treat a variety of cancers, including primary epithelial ovarian, colon, non-small cell lung and metastatic breast cancers. Its mechanism of action is through stabilization of microtubules which promotes polymerization of tubulin, causing cell death by disrupting the dynamics necessary for cell division (Lin et al., 2012).

It disrupts the dynamic equilibrium within the microtubule system and blocks cells in the late G₂ phase and M phase of the cell cycle, thereby inhibiting cell replication (Danhier et al., 2009).

It has been reported that Taxol[®] induces Bcl2 phosphorylation followed by apoptosis (Danhier et al., 2009).

Due to its high hydrophobicity and poor solubility in aqueous solutions, PTX is normally formulated with high percentages of additives such as Cremophor EL (polyethoxylated castor oil), which can cause high incidences of adverse effects including hypersensitivity reactions, neurotoxicity, myelosuppression and allergic reactions (Brannon-Peppas and Blanchette, 2004).

Numerous investigations have shown that both tissue and cell distribution profiles of anticancer drugs can be controlled by their entrapment in submicronic colloidal systems. Accordingly, a number of alternative formulations were investigated for the solubilization of PTX, including liposomes, microspheres, nanoparticles and polymeric micelles. The rationale behind this approach is to increase antitumor efficacy while reducing systemic side effects. Nanoparticulate drug delivery systems have been studied

for several decades now, and many of the features that make them attractive drug carriers are well known. Moreover, nanoparticles can escape from the vasculature through the leaky endothelial tissue that surrounds the tumor and then accumulate in certain solid tumors by the so-called Enhanced Permeation and Retention (EPR) effect (Danhier et al., 2009).

To avoid the toxicities associated with Cremophor EL, a biologically interactive, nanometer-sized albumin-bound PTX particle Abraxane, has been developed (hydrodynamic diameter of ca. 110 nm), which uses the unique properties of albumin, a natural carrier of lipophilic molecules in humans (Kratz, 2008).

Abraxane contains nanoparticles of paclitaxel bound to human serum albumin, the paclitaxel being in an amorphous state. Albumin is known to mediate endothelial transcytosis of plasma constituents and *in vitro* studies have demonstrated that the presence of albumin enhances paclitaxel transport across endothelial cells (www.has-sante.fr/portail/uploaddocs/applicationpdf2011-01abraxane_ct_7088.pdf 10.02.2013).

Abraxane[®], has recently been approved by the FDA for recurrent metastatic breast cancer (Danhier et al., 2009) in 2005 and for non small cell lung cancer in 2012 (<http://www.fda.gov/Drugs/InformationOnDrugs/ApprovedDrugs/ucm323668.htm> 16.05.2013).

Materials and Methods

Cell culture

The HeLa cell line used in this experiment was obtained from European Cell Culture Collection (CCL).

¹Department of Biology, Institute of Science, ²Department of Biology, Faculty of Science, Istanbul University, Istanbul, Turkey *For correspondence: nrcngrss@gmail.com

Cells 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), Amphotericine B (Sigma, USA) and 2 mM glutamine at 37°C in humidified atmosphere of 5% CO₂ in air. The pH of the medium was adjusted to 7.4 with NaHCO₃.

Drug treatments

Drug concentrations which were applied were determined based on previous *in vitro* and clinical studies. At first as a 1000 nM stock solution was prepared with M-199 supplemented with 10% FBS. Three different doses were obtained by dilution of stock solution. These doses were determined as dose 1 (D₁)=10 nM, dose 2 (D₂)=50 nM and dose 3 (D₃)=100 nM. The experiments were tested by using these three doses and these were treated to HeLa cells in the time periods of 24, 48 and 72 hours.

Preparing and application of ³H-Thymidine

Nine ml deionized water was added to a vial containing 1 mCi/ml ³H-Thymidine (TRA-120, Amersham, England) and stock solution was prepared. Then 1 mCi/ml solution was diluted to 1 µCi/ml with cell culture medium. The cells will be labelled with this solution.

The optimal dose of drug (D₂=50 nM) applied to HeLa cells. Cells were exposed to Abraxane in 24, 48 and 72 h for the labelling index. At the end of these periods, to investigate the labeling index parameter cells were incubated in medium containing 1 µCi/ml ³H-thymidine for 20 min. and cells were labelled. Then fixation was carried out.

Labelling index (LI) analysis

Autoradiograms were stained with Giemsa stain at 16°C for 3 min. For each drug concentration and time period of each application 3000 cells were counted under light microscope and percentage of cells labelling was calculated.

Mitotic index (MI) analysis

Mitotic index were studied by the methods of Feulgen. Before the cells were treated with Feulgen, they were prepared with 1 N HCl at room temperature for 1 minute and then hydrolized with 1 N HCl for 10.5 minutes at 60°C. After slides were treated with Feulgen, they were rinsed for few minutes in distilled water and stained with 10% Giemsa stain solution pH 6.8, for 3 minutes and washed twice in phosphate buffer. After staining, the slides were rinsed in distilled water. And then the slides were air dried. At last mitotic index were calculated by counting metaphases, anaphases and telophases for each tested drug concentration and control. At least three thousands cells were examined from each slide for mitotic index.

Apoptotic index (AI) analysis

The apoptotic index (AI) was found using fluorescence microscope. The AI is the percentage of cells undergoing apoptosis. For the determination of the AI, cells were fixed under methanol and stained with 4'-6 diamidine-2 phenylindol (DAPI). Following extensive washing in

phosphate-buffered saline (PBS), slides were scored in double blind under the fluorescence microscope. The AI was determined on a microscope field of at least 100 areas/ each experimental points by the same scorer.

Statistical analysis

The value of growth rates, mitotic index and apoptotic index were evaluated relative to control and each other. For this reason, values obtained from all experimental groups were analyzed using one-way ANOVA test. The significance between control and experimental groups was determined by DUNNETT's test and significance between experimental groups was determined by Student's t-test.

Results

Determination of optimal dose with mitotic index analysis

In this study three different doses of Abraxane (D₁=10nM, D₂=50nM, D₃=100nM) were applied to HeLa cell culture for 24, 48 and 72 h and anti mitotic effects of these doses were evaluated with mitotic index analysis. As a result of this analysis, cells which were in mitosis and which are not were counted (Table 1). All the differences between control and experimental groups were statistically significant (p<0.001). Additionally it was seen that there were a significant difference between experimental groups (p<0.001) 24 hours after drug administration, mitotic index values 5.24% for D₁, 3.18% for D₂ and 3.05% for D₃ compared with control group which was considered 100% (Figure 1).

Administration of optimum dose (24-72 h)

Among the three different Abraxane doses administered to the cells for 24, 48 and 72 h, mitotic index value of dose 2 which is for the purpose of the experiment was determined at 24, 48 and 72 h.

After treatment of HeLa cell culture with D₂, mitotic index values of control and experimental groups were counted for each hour. Following the detection of mitotic index values, graphics were generated by these values. In the statistical analysis it was determined that there was a significant difference between control and experimental groups at 24-72 h (p<0.001) (Table 2).

According to the mitotic index values, while the mitotic index of the control group was considered 100%,

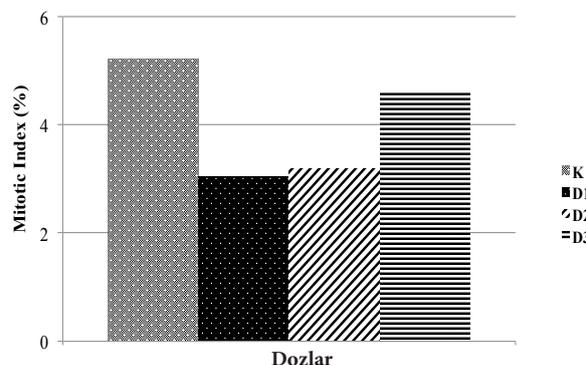


Figure 1. Mitotic Index Values of HeLa Cells Treated with 3 Different Doses of Abraxane for 24 h (D₁=10nM, D₂=50nM, D₃=100nM) (p<0.001)

Table 1. Mitotic Index Values of HeLa Cells Treated with 3 Different Doses of Abraxane for 24 h ($D_1=10\text{nM}$, $D_2=50$, $D_3=100\text{nM}$)

Groups	MI (%)
Control	5.24±2.32*
D_1	3.05±1.85
D_2	3.18±1.84
D_3	4.61±1.87

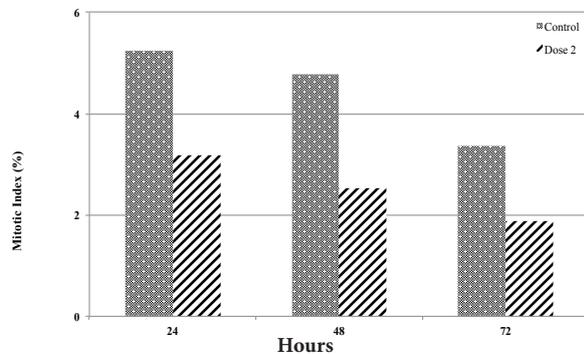


Figure 2. Mitotic Index Values of HeLa Cells Treated with D_2 Dose of Abraxane ($D_2=50\text{nM}$) for 0-24 h ($p<0.001$)

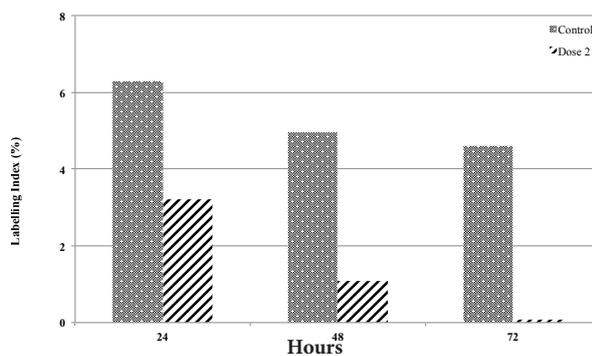


Figure 3. Labelling Index Values of HeLa Cells Treated with D_2 Dose of Abraxane ($D_2=50\text{ nM}$) for 0-24 h ($p<0.001$)

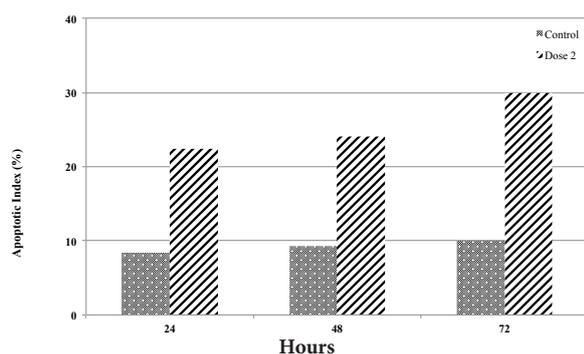


Figure 4. Apoptotic Index Values of HeLa Cells Treated with D_2 Dose of Abraxane ($D_2=50\text{ nM}$) for 0-24 h ($p<0.001$)

the mitotic index values of experimental groups compared with control group were detected as 3.18% for 24 h, 2.53% for 48 h and 1.89% for 72 h (Figure 2).

According to the mitotic index analysis which was performed with administration of Abraxane 24, 72 and 72 h time over the period, it was concluded that compared to control the mitotic index of HeLa cells was significantly decreased in a dose dependent manner ($p<0.001$).

Determination of DNA Synthesis with ^3H Thymidine

Morphological Evaluation: The effect of Abraxane on the DNA synthesis of HeLa cells determined by counted the cells labelling with ^3H thymidine. The labelled cells were evaluated with Feulgen method and Giemsa staining under light microscope.

Labelling Index (LI): After administration of D_2 dose ($D_2=50\text{ nM}$) of Abraxane for 24, 48 and 72 h, preparations were prepared, Feulgen method and Giemsa staining were applied to them. For all experimental groups, per 100 cells in at least 30 field were counted. For evaluation of LI, normal cells and the labelled cells were counted.

In all experimental groups, LI value of 24 h were detected as 6.28% for control, % 3.21% for D_2 , 48 h 4.96% for control, 1.08% for D_2 and 72 h 4.62% for control, % 0.08% for D_2 (Table 3). There were a significant difference between control and experimental groups ($p<0.001$). In addition, statistically there were a significant difference between all experimental groups ($p<0.001$). The values of the LI that belongs D_3 administrated to cells for 24-72 h time period was shown in Figure 3. Statistical analysis showed that for LI values there were a significant difference between control and experimental groups ($p<0.001$).

Apoptosis

Morphological Evaluation: Expected apoptotic morphological changes were shrinkage of cell membrane, blebbing of cell membrane, nuclear condensations; thus cell deformation and formations of apoptotic bodies. Afterwards, nucleus breaks into fragments (Yang et al., 2010). In our study this apoptotic morphological changes were detected on Abraxane treated HeLa cells by light microscope and fluorescence microscope.

Apoptotic Index (AI): For the 24, 48 and 72 hours after administration of $D_2=50\text{ nM}$ dose of Abraxane, approximately 200 cells were counted for both control and experimental groups. The value of AI in all groups for 24 h were detected as 8.38% for control, 22.37% for D_2 , for 48 h 9.27% for control, 24.02% for D_2 and for 72 h 10.6% for control, 30% for D_2 (Table 4).

All the differences between control and experimental groups were statistically significant ($p<0.001$). Additionally it was seen that there were a significant difference between experimental groups ($p<0.005$). 24-72 h AI values of D_2 dose was shown in Figure 4. In the statistical analysis, it was determined that there was a significant difference between control and experimental groups ($p<0.001$).

Mitosis

Morphological Evaluation: Late prophase, metaphase, anaphase and telophase of mitosis in HeLa cells were evaluated with Feulgen method and Giemsa staining under light microscope. Administration of D_2 of Abraxane caused of tripolar metaphase which is not occur normally.

Mitotic Index (MI): After administration of D_2 dose ($D_2=50\text{nM}$) of Abraxane for 24, 48 and 72 h, preparations were prepared, Feulgen method and Giemsa staining were

applied to them. For all experimental groups, per 100 cells in at least 30 field were counted. For evaluation of MI, normal cells and the cells that were in late prophase, metaphase, anaphase and telophase were counted.

In all experimental groups, MI value of 24 h were detected as 5.24% for control, 3.18% for D₂, 48 h 4.79% for control, 2.53% for D₂ and 72 h 3.36% for control, 1.89% for D₂ (S3). There were a significant difference between control and experimental groups (p<0.001). In addition, statistically there were a significant difference between all experimental groups (p<0.001). The values of the MI that belongs D₃ administrated to cells for 24-72 h time period was shown in S4. Statistical analysis showed that for MI values there were a significant difference between control and experimental groups (p<0.001).

Discussion

Surgery, radiotherapy and chemotherapy are conventional methods used in cancer treatment. Because these methods have some limitations, it is difficult to cure the disease completely. In recent years, to overcome these limitations and also to increase the efficiency of the therapies, new methods are being developed. In this context, nanotechnology is a promising approach. Therefore, cancer nanotechnology has become an important field. Some applications used in the field of cancer nanotechnology include novel nanodrugs that decrease the adverse effects of conventional cancer drugs and increase their therapeutic efficacy, gold nanoparticles which increase the sensitivity to radiotherapy and nanoparticles used in thermal ablation therapy among many others (Topcul and Cetin, 2013).

In our study, we researched the antiproliferative effect of Abraxane on HeLa cell line by evaluating the parameters of cell kinetic; labelling index, mitotic index and apoptotic index.

It is reported that, Paclitaxel and Abraxane exhibited *in vitro* inhibition of H295R and SW-13 cells (Demeure et al., 2008).

It is observed that Abraxane has antitumoral effect on OS-732 cell line and arrest cell division G₂/M phase *in vitro* (Yang et al., 2010)

In a study which is performed by Zhang et al. (2013), the effect of Abraxane on gastric cancer which is the second common cause of cancer related death worldwide and lacks highly effective treatment for advanced disease was researched. In that study, human gastric cancer cell lines AGS, NCI-N87 and SNU16 were studied. Abraxane inhibited cell proliferation in AGS and 49 nM in NCI-N87 cells after 72-hour treatment, which was lower than oxaliplatin and epirubicin.

Desai et al. (2006) studied the antitumor activity, intratumoral paclitaxel accumulation, and endothelial transport for Abraxane and Cremophor-based paclitaxel. Antitumor activity and mortality were assessed in nude mice bearing human tumor xenografts [lung (H522), breast (MX-1), ovarian (SK-OV-3), prostate (PC-3), and colon (HT29)] treated with ABI-007 or Cremophor-based paclitaxel. Intratumoral paclitaxel concentrations (MX-1-tumored mice) were compared for radiolabeled Abraxane

and Cremophor-based paclitaxel. Both Abraxane and Cremophor-based paclitaxel caused tumor regression and prolonged survival; the order of sensitivity was lung>breast congruent with ovary>prostate>colon. At equitoxic dose, the Abraxane-treated groups showed more complete regressions, longer time to recurrence, longer doubling time, and prolonged survival. At equal dose, tumor paclitaxel area under the curve was 33% higher for Abraxane versus Cremophor-based paclitaxel, indicating more effective intratumoral accumulation of Abraxane (Desai et al., 2006).

Although there have been previous efforts to optimize dose intensity or change the chemotherapy protocol for osteosarcoma, long-term survival has not been markedly improved during the past 15 years. Nude mice bearing established OS-732 human osteosarcoma received varying doses of Adriamycin, paclitaxel and Abraxane to assess tumor growth inhibition. Administration of Abraxane showed a tumor inhibitory rate of 98.8% (tumor weight), which was significantly higher than adriamycin (46.1%, tumor weight,) and paclitaxel (40.8%, tumor weight). The antitumor effect of Abraxane was demonstrated in osteosarcoma xenografts *in vivo* (Yang et al., 2012).

According to the data obtained from our results, 50nM concentration of Abraxane that applied on HeLa cells caused to decrease on mitotic index especially in 72 h. It demonstrated us that Abraxane inhibits cell division or undergoes cells to death.

Abraxane demonstrated greater efficacy and a favorable safety profile compared with standard paclitaxel in this patient population. The improved therapeutic index and elimination of corticosteroid premedication required for solvent-based taxanes make the novel albuminbound paclitaxel Abraxane an important advance in the treatment of metastatic breast cancer (Gradishar et al., 2005).

A study of MCF-7 human breast cancer cells was undertaken to ascertain the degree of apoptosis induction by paclitaxel. Paclitaxel (0-20 ng/ml) caused concentration-dependent increases in morphologically identifiable apoptotic cells (up to 43% of cell population) and cells with DNA strand breaks (up to 38%), a commonly cited marker of apoptosis. Maximal DNA strand breakage occurred after 16 hr of exposure to paclitaxel and maximal apoptotic-appearing cells occurred after 24 hr. The remaining non-apoptotic paclitaxel-exposed cells were growth arrested in G₂ (Saunders et al., 1997).

In our study, implementation of optimal dose D₂=50 nM increased the ratio of apoptotic cells from 8.38% to 22.37% in 24 h and time dependent growing proportion of apoptotic cells was also observed. Saunders and his colleagues study of breast cancer cells and our study supporting each other.

In a multicenter Phase II trial to evaluate the safety and efficiency of Abraxane, which is designed to increase the concentration of active drug in tumor, patients with non small cell lung cancer were used. In this study, 43 patients were included and overall response rate was 16% and the disease control rate of 49% respectively. The median time to progression was 6 months and median survival was 11 months. The possibility of not progress within 1 year was 13%, 1 year survival probability is 45%. Although there

was no premedication, hypersensitivity reactions have not been reported (Green et al., 2006).

Sparreboom et al. (2005) compared the preclinical and clinical pharmacokinetic properties of paclitaxel formulated as a Cremophor-free, albumin-bound nanoparticle (Abraxane) and formulated in Cremophor-ethanol (Taxol). The volume of distribution at steady state and clearance for paclitaxel formulated as Cremophor-free nanoparticle Abraxane were significantly greater than those for paclitaxel formulated with Cremophor (Taxol) in rats. Fecal excretion was the main elimination pathway with both formulations. Consistent with the preclinical data, paclitaxel clearance and volume of distribution were significantly higher for Abraxane than for Taxol in humans (Sparreboom et al., 2005).

In a study with mice bearing syngeneic ovarian or mammary carcinomas, Nab-paclitaxel showed single-agent antitumor efficacy against both tumor types and acted as a radiosensitizer. Combined with radiation, nab-paclitaxel produced supra-additive effects when given before radiation. Nab-paclitaxel significantly increased radiocurability by reducing the dose yielding 50% tumor cure (TCD50). Tumor histology following nab-paclitaxel treatment was characterized by pronounced necrotic and apoptotic cell death and mitotic arrest. Nab-paclitaxel did not increase normal tissue radioresponse (Wiedenmann et al., 2007).

According to the findings of our study that, increased time and labeling index values are examined especially during the 72-hour concentration, percentage of synthesis phase of HeLa cells treated with 50nM dose of Abraxane significantly reduced and almost all of the cells could not undergo to the synthesis phase.

Abraxane is well tolerated in women with gynecologic cancer who have experienced a paclitaxel-associated hypersensitivity reaction (Fader and Rose, 2009).

Results of our study, in accordance with the above-discussed studies showed that Abraxane has a significant antiproliferative effect on cervical cancer cells.

In conclusion, our study that, examined the cell kinetic parameters of cancer cells, indicating compliance with the finding of above-described researchers studies. Apply on the HeLa cells 50nM dose of Abraxane was determined to be caused an increase in apoptotic index and a decrease in mitotic and labelling index of tumor cells. Considering the above-mentioned pre-clinical and clinical studies, it is apparent that, Abraxane will provide an effective treatment on several types of cancer compared with non-nanotechnological derivative.

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