Effects of epirubicin and daunorubicin on cell proliferation and cell death in HeLa cells

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Abstract

Epirubicin and daunorubicin, antibiotics which are derivative of anthracyclines, are especially used on widespread tumors. In this study, effects on cell growth and cell death were examined by MTT method applying in vitro doses of epirubicin, daunorubicin and epirubicin+daunorubicin to human servical carcinoma derived HeLa cell line. According to the results obtained from the experiments it was determined that daunorubicin has more cytotoxic effect than epirubicin depending on the periods of drug exposure. In whole experiment groups, the most effective result was detected in epirubicin+daunorubicin combination (p< 0.001).

Key words: Epirubicin, Daunorubicin, HeLa cells, cell proliferation, cell death, MTT method.

Epirubisin ve daunorubisinin hücre çoğalması ve hücre ölümü üzerine etkilerinin HeLa hücrelerinde araştırılması

Özet

Antrasiklin türevi epirubisin ve daunorubisin, özellikle yaygın tümörlerde sıklıkla kullanılan antibiyotiklerdir. Bu çalışmada, epirubisin, daunorubisin ve epirubisin+daunorubisinin in vitro dozları insan serviks kanseri kökenli HeLa hücre soyuna uygulanarak, hücre çoğalması ve hücre ölümü üzerine olan etkileri MTT yöntemi ile araştırıldı. Deneylerde elde edilen sonuçlara göre, ilaç uygulama süresine bağlı olarak daunorubisinin epirubisinden daha sitotoksik bir etkiye sahip olduğu belirlendi. Tüm deney grupları içerisinde en etkili sonuç, epirubisin+daunorubisin kombinasyonunda elde edildi (p< 0.001).

Anahtar sözcükler: Epirubisin, Daunorubisin, HeLa hücreleri, hücre çoğalması, hücre ölümü, MTT yöntemi.

Introduction

Cancer chemotherapy, a third treatment method beside the surgical operation and radiotherapy, is carried out applying various drugs to especially widespread tumors (Zhang et al., 1992). Cancer chemotherapy is used for dynamics processes related with proliferation or rate of growing smaller in tumor, considering the origin of cell groups which form tumor. Beside this, it should be evaluated action mechanism, toxicity and side effects of drug. Cytotoxics materials used on cancer chemotherapy more affect the cell in the process of division in comparison with the cell in rest attitude (Robert and Gianni, 1993; Topçul et al., 2002).

In recent years, cancer chemotherapy has largely utilized of recorded new developings related with cell kinetics. Results from the studies to explain effects on cell level of different types of radiations, various chemicals, hormones and cytotoxic drugs make the studies concerning cell proliferation and cell death
important in direction of establishing period of cell cycle and periods of phases (Skladanowski and Konopa, 1993; Chan and Chan, 1999).

Epirubicin (EPI) and Daunorubicin (DAU), the antitumor antibiotics derivative of anthracyclines, are often used on especially widespread tumors. Cell culture studies indicates that EPI and DAU rapidly diffuse inside the cell, localise in nucleus and inhibit synthesis of nucleic acid (Greg et al., 1993; Mansilla et al., 2003; Ralph et al., 2003). In this study, it was investigated both alone and combined effects of EPI and DAU on cell growth and cell death on HeLa cells. It was also aimed that being more understood the effects of cytotoxic drugs and possessing attributes of being preliminary study related to using separate or combined in clinic.

Material and methods

Cell line

Tumoral cell line used in our experiments was HeLa (CCL 2) cells that was taken from human servical carcinoma in 1951 and that has been continously grown in cell culture since that date. These cells were obtained to our laboratory by Tokio Technology Institute and was grown regularly by doing passage twice a week.

Cell culture

Cells were cultured in Minimum Essential Medium (MEM, Sigma) containing 10% fetal bovine serum (FBS, Gibco Lab.) and 1% penicillin-streptomycin at 37°C in an atmosphere containing 5% CO₂. Culture medium was changed every 2 or 3 days. Cells were washed with Hank’s balanced salt solution (HBSS) and harvested using 5% trypsin (Gibco Lab.) solution. Then cells were centrifuged at 1500 rpm for 3 minutes. Supernatant was discarded and pellet was diluted with MEM. Cells were seeded 10.000 cells/well in 96 well plates. After these cells incubate at 37°C for 24 hours, experiments were done.

Experimental design

10 mg EPI (Farmorubicin, Carlo Erba) and 20 mg DAU (Daunorubicina, Carlo Erba) were dissolved in MEM as a 1mg/ml stock solution supplemented with 10% FBS. The pH of the drug solution was adjusted to 7.4 with NaOH. All assays testing EPI and DAU were protected from light. The required final concentrations (0.01 µg/ml, 0.1 µg/ml, 0.5 µg/ml, 1 µg/ml and 5 µg/ml) were obtained by diluting aliquots of the stock solution in cell culture medium (MEM) supplemented with 10% FBS. It was determined inhibitory concentration 50% (IC₅₀) doses by applying above mentioned doses of both EPI and DAU in HeLa cell culture. The experiments tested by using IC₅₀ doses were carried out in 4 groups such as Control, EPI, DAU and EPI+DAU combinations. With this aim, determined IC₅₀ doses of drugs were treated to HeLa cells in the time periods of 0, 2, 4, 8, 16, 24 hours. Each experiment was performed on at least three separate occasions.

Chemical

EPI (4'-epidoxorubicin), an anthracycline, is a doxorubicin stereoisomer, possessing the L-arabino instead of the L-lyxo configuration of the sugar moiety (Figure 1). In EPI therefore the hydroxyl group on the sugar moiety, possessing the stable 'C₄ conformation, has an aquatorial orientation (Arıcan Özcan and Topçul, 2003).

DAU (4-demethoxydaunorubicin) is meant an antibiotic of the rhodomycin group, originally isolated from fermentation broths of Streptomyces peucetius and Streptomyces coentleonibidus and its acid complexes particularly its hydrochloride complex. DAU is a glycoside formed by a tetracyclic aglycone daunomycinone and an amino sugar daunosamine (Keprtova et al., 1993) (Figure 1).

Figure 1: Structural formulae of EPI and DAU.

Daunorubicin: —OCH₃ ---H —OH —H
Epirubicin: \(-\text{OCH}_3\) \(-\text{OH}\) \(-\text{H}\) \(-\text{OH}\)

**MTT analysis**

The cell viability was determined by the Mosmann’s MTT assay with minor modifications (Mosmann, 1983; Fischer et al., 1999; Mc Daid and Johnston, 1999). MTT analysis were applied to identify cytotoxicity that was formed by drugs in time intervals for 0-24 hours. Therefore, MTT stock solution (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma) as a 5 mg/ml was prepared in phosphate-buffered saline (PBS, Sigma). At the end of the above mentioned treatment intervals, 20 µl MTT solution was added to each culture on condition that using final concentration was 0.5 mg/ml. After 4 hours incubation, the liquid on cells was removed, and 200 µl dimethyl sulfoxide (DMSO) was added to each well on formed formazan crystals. To dissolve formazan crystals formed in cell, 96 well plates was stayed in shaker incubator for 30 minutes. Absorbances were measured at 570nm using an UV-160 spectrophotometre (Shimadzu).

**Statistical analysis**
The statistical significance between the control and treatment groups was determined by Student-t test. In all cases, a P value less than 0.001 was considered significant.

**Results**

The effects of EPI and DAU on cell proliferation and cell death were investigated in this study. After 0.01 mg/ml, 0.1 mg/ml, 0.5 mg/ml, 1 mg/ml, 5 mg/ml doses of EPI were applied to HeLa cells for 24 hours respectively, the absorbances of drug treated cells were reduced to $7.1 \times 10^{-2}$, $5.7 \times 10^{-2}$, $4.8 \times 10^{-2}$, $3.8 \times 10^{-2}$, $2.8 \times 10^{-2}$ compared that of control absorbance as $11.3 \times 10^{-2}$. With 0.01 µg/ml, 0.1 µg/ml, 0.5 µg/ml, 1 µg/ml, 5 µg/ml doses of DAU treatment for 24 hours respectively, absorbances of HeLa cells were reduced to $3.4 \times 10^{-2}$, $2.4 \times 10^{-2}$, $1.5 \times 10^{-2}$, $1.4 \times 10^{-2}$, $1.2 \times 10^{-2}$ compared that of control absorbance as $10.9 \times 10^{-2}$ (Figure 2).

When HeLa cells were treated with 0.01 µg/ml, 0.1 µg/ml, 0.5 µg/ml, 1 µg/ml, 5 µg/ml doses of EPI for 48 hours respectively, the absorbances of drug-treated cells were measured $4.8 \times 10^{-2}$, $3.55 \times 10^{-2}$, $0.25 \times 10^{-2}$, $0.25 \times 10^{-2}$ and $0.05 \times 10^{-2}$ compared that of control absorbance as $5.85 \times 10^{-2}$. After the same doses of DAU were applied to cells for 48 hours respectively, the absorbances of HeLa cells were reduced to $2 \times 10^{-2}$, $0.4 \times 10^{-2}$, $0.3 \times 10^{-2}$, $0.3 \times 10^{-2}$, $0.15 \times 10^{-2}$ compared that of control absorbance as $5.8 \times 10^{-2}$ (Figure 3).

When the HeLa cells were treated for 24 and 48 hours with 0.01 µg/ml, 0.1 µg/ml, 0.5 µg/ml, 1 µg/ml, 5 µg/ml of EPI and DAU, their growth and viability were significantly decreased in dose- and time-dependent manners ($p<0.001$). It was determined that IC$_{50}$ value of EPI is 0.1 µg/ml, IC$_{50}$ value of DAU is 0.01 µg/ml for 24 hours treatment and IC$_{50}$ value of EPI is 0.2 µg/ml, IC$_{50}$ value of DAU is 0.008 µg/ml for 48 hours treatment (Figures 4, 5).

Detected IC$_{50}$ values of the first experiment of EPI and DAU were used to establish effect on cell viability for 0-24 hours. IC$_{50}$ values of EPI and DAU (0.1 µg/ml and 0.01 µg/ml, respectively) were applied for 24 hours. Applying these IC$_{50}$ values to HeLa cells, it was provided to decrease cell viability in time-dependent manner (Figure 6). In all experiment groups, the most effective result was detected in EPI+DAU treatment. Inhibition percentage of cell proliferation, which were calculated by taking control value as 100%, was given in Figure 7. In combined treatment, cell viability was influenced by >50% inhibition at 8 hours and reduced to 20% at 24 hours.

**Discussion**

EPI and DAU are the anthracycline antibiotics that have been examined to determine of their cytotoxities on HeLa cells in this study. Anthracycline antibiotics

![Figure 6](image1.png)

**Figure 6:** Effect of IC$_{50}$ values of EPI and DAU either alone or in combination in HeLa cells for 0-24 hours.

![Figure 7](image2.png)

**Figure 7:** Effect of IC$_{50}$ values of EPI and DAU either alone or in combination on vitality in HeLa cells for 0-24 hours.
have been in use extensively in the treatment of widespread tumors. It is suggested that they form a complex with DNA by intercalation between DNA strands (Piagram et al. 1972), thus inhibiting DNA replication and transcription (Di Marco et al., 1971; Glisson and Ross, 1987), and inducing DNA fragmentation with inhibition of repair (Lee et al., 1974). Intercalation appears to interfere with the topoisomerase-DNA ‘cleavable complex’ and is emerging as a potentially important mechanism of action of the anthracyclines (Spadari et al., 1986; Glisson and Ross, 1987; Mouridsen et al., 1990). Finally, the anthracyclines have been shown to induce apoptotic cell death (Ling et al., 1993; Jaffrezou et al., 1996), although this is likely to be the final cellular response to upstream events such as inhibition of topoisomerase II (Gewirtz, 1999).

EPI is active against a range of tumors and is widely used in the treatment of women with early or advanced breast cancer, administered either alone or in combination with other anticancer agents (Plisker and Faulds, 1993). DNA synthesis inhibition, free radical formation and lipid peroxidation, DNA binding and alkylation, DNA cross-linking, interference with DNA strand separation and helicase activity, direct membrane effects, and the initiation of DNA damage via the inhibition of topoisomerase II are the mechanisms responsible for the antiproliferative and cytotoxic effects of the daunorubicin (Gewirtz, 1999). Maximal lethal effects of EPI were demonstrated in the S and G$_2$ phases of the cell cycle in murine and human tumour cell lines (Hill and Whelan, 1982). EPI has been shown to inhibit proliferation of all neuroblastoma cell lines by 69 to 78% relative to controls (Rocchi et al., 1987), of a human alveolar rhabdomyosarcoma cell clone (Lollini et al., 1989), and of haemopoietic progenitor cells from several human leukemic cell lines in liquid culture (Bagnara et al., 1987).

It is demonstrated that inhibition of DNA and RNA synthesis in HeLa cells over a concentration range of 0.2 through 2 $\mu$M DAU (Di Marco et al., 1965). It is also found that DAU concentrations of at least 4 $\mu$M were required before effects on DNA synthesis were detected in Ehrlich ascites tumor cells (Dano et al., 1972). IC$_{50}$ value was determined as 1 $\mu$M after a 2 hour pulse incubation of HL60 cells with DAU followed by 3-day incubation (Masquelier and Vitos, 2004).

In this study we found that DAU’s cytotoxic effect had more available than EPI’s cytotoxicity for HeLa cells. Besides, we demonstrated that IC$_{50}$ doses of EPI and DAU for 24 hours. Detected IC$_{50}$ values referred to determine cytotoxicity in the time periods of 0, 2, 4, 8, 16, 24 hours. It was found that cell viability decreased with IC$_{50}$ values in time-dependent manner. The most effective loss of vitality in HeLa cells was found in EPI+DAU combination. Cell viability in this group was reduced to 20% at the end of 24 hours. After conducting more in vivo and in vitro experiments in various cell groups, the results obtained in this study could play an important role as a resource when designing tumor therapy programs.

References

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