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Full Length Research Paper

Evaluation of the cytotoxicity interactions between epirubicin and daunorubicin in HeLa cell cultures

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Epirubicin and Daunorubicin, antibiotics which are derivative of anthracyclines, are used on cancer chemotherapy. In this study, Epirubicin and Daunorubicin effects on cell kinetics parameters were examined both single and in combination on HeLa cell culture that was taken from human servical carcinoma. The experiments tested by using IC_{90} doses of Epirubicin and Daunorubicin (0.5 µg/ml and 0.1 µg/ml, respectively) were applied for 24 and 48 h. Cell kinetics parameters such as growth rate (WST-1 colorimetric assay), mitotic and apoptotic index were applied to identify cytotoxicity that was formed by drugs. In addition, DNA degradation was examined in agarose gel electrophoresis. The cytotoxic effect of drugs appeared significant decline of growth rate and mitotic index, and significant increase of apoptotic index of HeLa cell line ($p<0.05$ - 0.001). The enhancement of the cytotoxicity to Daunorubicin was closely associated with the DNA degradation.

Key words: HeLa cells, cell kinetics, DNA, apoptosis, epirubicin, daunorubicin.

INTRODUCTION

The anthracycline antitumor antibiotics occupy an important role in the field of cancer chemotherapy (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. Besides 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. In recent years, cancer chemotherapy has largely utilized recorded new developings related with cell kinetics (Zeng et al., 2000; Fogli et al., 2002; Arıcan, 2005).

Epirubicin (EPI) and Daunorubicin (DAU), the antitumor antibiotics derivative of anthracyclines, are often used on especially widespread tumors. Those are the epimer of the anthracycline antibiotic doxorubicin, which has been used alone or in combination with other cytotoxic agents in the treatment of a variety of malignancies. EPI and DAU are a cell cycle phase non-specific anthracyclines, with maximal cytotoxic effects in the S and G2 phases (Özcan et al., 1997). Cell culture studies indicate that EPI and DAU bind to DNA, RNA, chromatin, and cell membrane, but their antitumor activity likely results from the inhibition of topoisomerase-II. DNA intercalation and stabilization occurs in the drug–nucleic acid-topoisomerase II ternary complex, which is referred to as the cleavable complex (Spadari et al., 1986; Greg et al., 1993; Nitsu et al., 2000).

In the present study, the *in vitro* cytotoxic activity of EPI and DAU and their combined effect of cytotoxicity were examined in HeLa cells. This study is also considered to be useful to better understand the function of cytotoxic agents could significantly repress the cell-killing activity as well as the general cytotoxic effect of drugs against tumor cells *in vitro*.

MATERIALS AND METHODS

Cell culture conditions

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.

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Cells were cultured in Minimum Essential Medium (MEM, Sigma) containing 10% Fetal Bovine Serum (FBS, Gibco Lab.) at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were washed with Hank's balanced salt solution (HBSS) and harvested using 0.25% trypsine (Gibco Lab.) for 3 min. Then cells were centrifuged at 1500 rpm for 3 min. Supernatant was discarded and pellet was diluted with MEM. Cells were seeded 30.000 cells/well in 96 well plates. After these cells incubate at 37°C for 24 h, experiments were done.

Drug treatments

10 mg EPI (Farmorubicin, Carlo Erba) and 20 mg DAU (Daunorubicina, Carlo Erba) were dissolved in MEM as a 1 mg/ml stock solution supplemented with 10% FBS. The pH of the drug solution was adjusted to 7.4 with $NaHCO_3$. All assays testing EPI and DAU were protected from light. It was determined inhibitory concentration 90% (IC_{90}) doses by applying above mentioned doses of both EPI and DAU in HeLa cell culture. The experiments tested by using IC_{90} doses (0.5 and 0.1 µg/ml, respectively) were carried out in 4 groups such as Control, EPI, DAU and EPI+DAU combinations. With this aim, determined IC_{90} doses of drugs were treated to HeLa cells in the time periods of 24 and 48 h.

Chemical

EPI (4'-epidoxorubicin), an anthracycline, is a doxorubicin stereoisomer, possessing the L-arabino instead of the L-lyxo configuration of the sugar moiety. In EPI therefore the hydroxyl group on the sugar moiety, possessing the stable $^1C_4$ conformation, has an aquatorial orientation. DAU (4-demethoxydaunorubicin) is meant an antibiotic of the rhodomycin group, originally isolated from fermentation broths of *Streptomyces peuicetius* 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; Styczynski et al., 2002).

WST-1 colorimetric assay

The effect of chemotherapeutic agents on the growth rate of HeLa cells were evaluated with the WST-1 assay kit (Premix WST-1, Takara, cat no: MK400). The WST-1 assay was applied to identify cytotoxicity that was formed by drugs for 24 and 48 h. Therefore, WST-1 reagent; 4 h later, the 96 well plates were read on a Mquant Microelisa reader, using a test wavelength of 490 nm, a reference wavelength of 690 nm.

Mitotic index analysis

Mitotic index (MI) was studied by the method of Feulgen. Before the cells were treated with Feulgen, they were prepared with 1 N HCl at room temperature for 1 min and then hydrolyzed with 1 N HCl for 10.5 min 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 min and washed twice in phosphate buffer. After staining, the slides were rinsed in distilled water. And then the slides were air dried. At last MI 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 MI.

Apoptotic index analysis

For the determination of the apoptotic index (AI), cells were fixed under methanol and stained with 4'-6-diamidine-2 phenylindole (DAPI). Following extensive washing in phosphate-buffered saline (PBS), slides were scored in double-blind under the fluorescence microscope. The AI represents the percentage of fragmented nuclei and was determined on a microscopic field of at least 100 area/ each experimental points by the same scorer.

DNA extraction

Genomic DNA was isolated from HeLa cells described in the protocol of manufacturer (Apopladder ExTM Kit, Takara, Cat. No. MK600). The DNA concentration of samples was measured with visible spectro photometer (GBC Cintra 20) at A_{260} and electrophoresed in 2% (w/v) agarose containing 1 µg/ml ethidium bromide.

Statistical analysis

The data were analyzed statistically using ANOVA. Dunnett's test (between control and treatment groups) and Student Newman-Keuls test (between treatment groups) were used for multiple comparisons. The data analyzed were those from a minimum of three independent experiments. For the statistical evaluation of the results, the significance was accepted at the probability level of $p < 0.05$.

RESULTS

Growth inhibitory effects of EPI and DAU

The absorbance of IC_{90} doses for EPI, DAU and EPI+DAU of HeLa cells are shown in Table 1. The absorbance value for drugs was higher for 24 than for 48 h in HeLa cells. When the HeLa cells were treated for 24 and 48 h with IC_{90} values of EPI and DAU, their growth and viability were significantly decreased in time-dependent manners according to control ($p < 0.001$) (Figure 1).

Mitotic index analysis of treated cells

The values of mitotic index for 24 and 48 h decreased in HeLa cells when treated with EPI, DAU and EPI+DAU, as seen in Table 2. These decreases were statistically significant when compared to control ($p < 0.001$). Another part of cytotoxic effect of these drugs are inhibition of mitotic index in HeLa cells.

Apoptotic index analysis of treated cells

The number of apoptotic cells of HeLa cell culture treated with EPI, DAU and EPI+DAU, as indicated in Table 3, revealed that drugs expose part of their cytotoxic effects as apoptosis induction. The values of apoptotic index also increased with increasing treatment time (Figure 2). The apoptotic cell number and apoptotic index values in DAU treatment was higher than in EPI treatment for both treatment times.

Table 1. Effect of IC_{90} values of EPI and DAU either alone or in combination for 24 and 48 h in HeLa cells.

Treatment groups	24 h	48 h
Control	$10 \times 10^{-2} \pm 1.68$	$11 \times 10^{-2} \pm 1.53$
EPI	$4.3 \times 10^{-2} * \pm 0.91$	$1.2 \times 10^{-2} * \pm 0.10$
DAU	$4.6 \times 10^{-2} * \pm 0.74$	$1.4 \times 10^{-2} * \pm 0.12$
EPI + DAU	$4.0 \times 10^{-2} * \pm 0.71$	$1.0 \times 10^{-2} * \pm 0.09$

Values are mean ± SD.
*p<0.001 (in comparison to control).

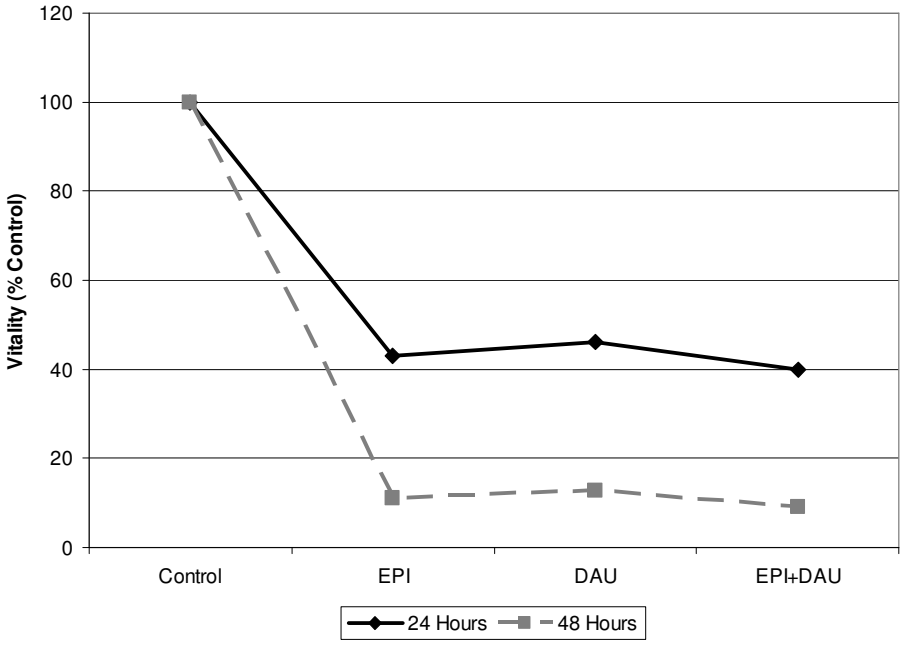

Figure 1. Cell growth inhibition of HeLa cell lines by EPI, DAU and EPI+DAU treatment for 24 and 48 h.

Table 2. Mitotic Index (MI) values of drug treatments.

Treatment groups	24 h	48 h
Control	2.44 ± 0.43	2.39 ± 0.53
EPI	1.63* ± 0.26	1.22* ± 0.12
DAU	2.01* ± 0.97	1.30* ± 0.27
EPI + DAU	1.04* ± 0.20	0.14* ± 0.01

Values are mean ± SD.
*p<0.001 (in comparison to control).

Table 3. Number of apoptotic cell of drug treatment in HeLa cell lines, given in ± SD.

Treatment groups	24 h	48 h
Control	3 ± 0.98	5 ± 1.44
EPI	19* ± 2.01	30* ± 2.77
DAU	25* ± 3.08	42* ± 3.48
EPI + DAU	20* ± 2.99	40* ± 3.01

Values are mean ± SD.
*p<0.001 (in comparison to control).

DNA profiles of treated cells

When experimental design (Control, EPI, DAU and EPI+DAU) for 24 h were applied to HeLa cell cultures, the concentrations of isolated DNA were measured 12.61, 13.52, 6.03 and 10.23 µg/ml, respectively. After the same design of drug treatments were applied for 48 h, the concentrations of isolated DNA were 13.16, 15.28, 7.33 and 10.42 µg/ml, respectively. As a result, high DNA degradation was determined in single DAU treatment for 24 and 48 h in HeLa cells. Agarose gel electrophoresis of DNA from HeLa cells treated with EPI, DAU and EPI + DAU showed in Figure 3 for 24 and 48 h, respectively. When the DNA levels of HeLa cells were compared in treatment groups, the most effective result was obtained

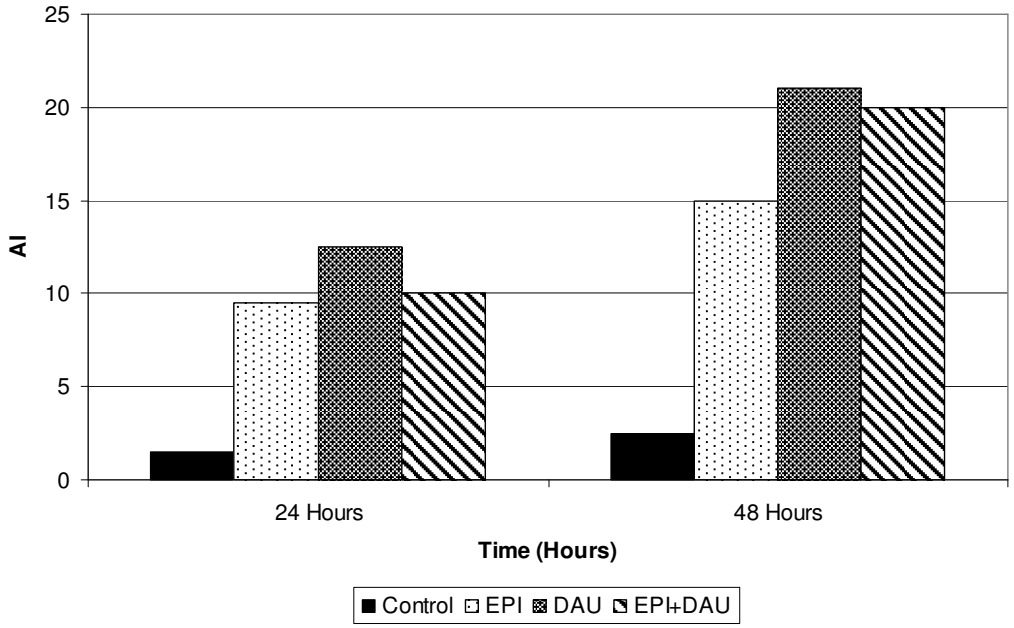

Figure 2. Apoptotic index (AI) of HeLa cell lines treated with EPI, DAU and EPI+DAU for 24 and 48 h.

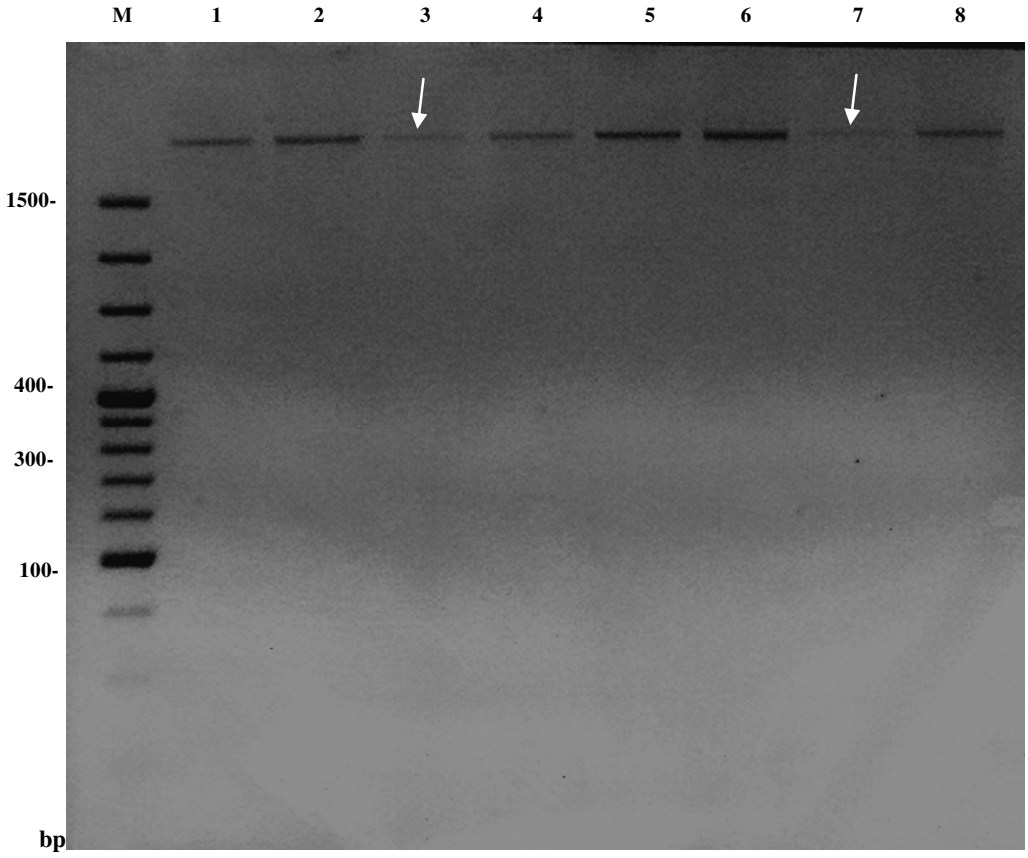

Figure 3. DNA profiles of EPI, DAU and EPI+DAU treatment groups in HeLa cells for 24 and 48 h. M, Marker (bp); 1, Control; 2, EPI; 3, DAU (white arrow) and 4, EPI+DAU for 24 h; 5, Control; 6, EPI; 7, DAU (white arrow) and 8, EPI+DAU for 48 h.

in single DAU treatment for 48 h.

DISCUSSION

Anthracycline antibiotics have been in use extensively in the treatment of widespread tumors (Rocchi et al., 1987; Robert and Gianni, 1993; Ralph et al., 2003). It is suggested that they form a complex with DNA by intercalation between DNA strands (Piagram et al., 1972). The anthracyclines have been shown to induce apoptotic cell death (Ling et al., 1993; Skladonowski and Konopa, 1993; Jaffrezou et al., 1996), although this is likely to be the final cellular reponse to upstream events such as inhibition of topoisomerase II (Gewirtz, 1999).

EPI and DAU are active against a range of tumors and are widely used in the treatment of women with early or advanced breast cancer, administered either alone or in combination with other anticancer agents (Plosker 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 and DAU were demonstrated in the S and G2 phases of the cell cycle in murine and human tumour cell lines (Hill and Whelan, 1982; Özcan et al., 1997).

In recent years, several studies have been reported on cytotoxicity and apoptotic activities of anticancer drugs of *in vitro* cultures (Bungu et al., 2006; Takara et al., 2006; Arıcan and Özalpan, 2007; Zhou et al., 2007).

In this study we examined the cytotoxicity of EPI and DAU with growth inhibition, AI and MI as the cell kinetic parameters. Also DNA profiles of treatment groups are screened at agarose gel electrophoresis. For this aim the cell cultures treated with both single and combined IC90 doses of EPI and DAU, which are determined in another study of ours, for 24 and 48 h time periods (Arıcan and Soy, 2005).

In estimated growth parameter the cytotoxicity of drugs caused a significant loss of vitality. EPI, DAU and EPI+DAU treatments decreased growth rate and MI of HeLa cell lines by increasing treatment time compared to controls ($p < 0.05$- $p < 0.001$). Treatment of EPI and DAU either alone or in bi-combination for 24 and 48 h decreased the growth rate according to (100%) control (43, 46, 40 and 10.9, 12.72, 9.09% respectively, Figure 1). The values of growth inhibition of the cells treated with these drugs alone for 24 and 48 h revealed that the decreases in cell number were statistically significant with respect to control ($p < 0.001$). The cytotoxic effect that occures increased in time dependent. There was no statistically difference for % vitality between treatment groups. Also the decreasing compared to control in MI, which examined in our experiments, increased in time dependent. When the differences of MI levels between different treatment groups and different times were analyzed, significant differences were found between treatments groups and between times ($p < 0.05$-$p < 0.001$). The values obtained for MI parameter were shown in Table 2. It was observed that depending on the drug treatments, inhibition of mitosis in HeLa cell cultures were increased, being of statistically significant for 24 h in all treatment groups. After 48 h treatment, when various drugs combinations were added to cultures, this significance levels seemed to continue ($p < 0.05$). This MI decrease in results supports the results that high doses of EPI and DAU, which are proper to S phase, are effective in M phase.

We examined the AI parameter to explain that the cytotoxicity, which occurs in experiment groups, is whether caused or not by apoptosis that induced by IC90 doses of drugs. As shown in our determined AI values, single DAU treated group has the highest AI value in all treatment groups ($p<0.05$). All AI values increased depends on treatment period. Determined DNA contents and profiles in agarose gel supported our AI results as in single DAU treatment.

In summary our study showed that DAU's cytotoxic effect had more available than EPI's cytotoxicity for HeLa cell cultures. Besides, we demonstrated that inhibition of growth rate and MI of EPI and DAU treatments for 24 and 48 h. Detected AI values referred to induce apoptosis in the time periods of 24 and 48 h. Results of studies on growth rate and MI effect of drugs in combination or single demonstrated that more detailed parameters must be investigated to evaluate the level of these effects. Nevertheless, the time-dependent increase observed in the level of cytotoxicity by combination treatment and decreased growth rate effect we demonstrated are in agreement with the results of previous studies. Particularly, demonstration of the cytotoxicity of single DAU treatment which appeared as increased of AI and DNA degradation is the most important finding of this study.

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