Effect of epirubicin on mitotic index in cultured L-cells

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Abstract

Cancer chemotherapy is an additional application to surgical operations and radiotherapy in the treatment of widespread tumors. An anthracycline-derived antibiotic, epirubicin (EPI) is one of the clinically used antineoplastic drugs. In this study the cytotoxic effects of EPI in transformed mouse fibroblasts (L-cell) were examined. EPI concentrations of 0.001 µg/ml, 0.01 µg/ml and 0.1 µg/ml were applied to the cells for 2, 4, 8, 16 and 32 hours. The results showed that EPI diminished mitotic index of L-cells depends upon time and applied concentrations. This decrease was found statistically significant in each treatment group when compared to control ($p<0.05$ - $p<0.001$).

Key words: Epirubicin, L-cell, transformed fibroblast, mitotic index, *in vitro*

Epirubisinin kültürdeki L-hücrelerinde mitotik indekse etkisi

Özet

Kanser kemoterapisi, yaygın tümörlerin tedavisinde cerrahi uygulama ve radyoterapinin yanında gerçekleştirilen ek bir uygulamadır. Antrasiklin türevi bir antibiyotik olan epirubisin (EPI) klinik olarak kullanılan antineoplastik ilaçlardan birisidir. Bu çalışmada, EPI in sitotoksik etkileri, transforme edilmiş fare fibroblastlarında (L-hücreleri) araştırıldı. EPI in 0.001 µg/ml, 0.01 µg/ml ve 0.1 µg/ml konsantrasyonları 2, 4, 8, 16 ve 32 saat süresince hücrelere uygulandı. Sonuçlar uygulanan zaman ve konsantrasyona bağlı olarak EPI in L-hücrelerinin mitotik indeks değerlerini düşürdüğünü gösterdi. Bu düşüş kontrol grubu ile karşılaştırıldığında, her bir deney grubunda istatistik olarak anlamlı bulundu ($p<0.05$ - $p<0.001$).

Anahtar sözcükler: Epirubisin, L-hücreleri, transforme edilmiş fibroblast, mitotik indeks, *in vitro*

Introduction

Of the cancer drugs in clinical use, the anthracyclines have a spectrum of antitumor activity and are clearly the most useful cancer drugs among the natural product (Chabner and Mayers, 1993).

Epirubicin (EPI) is the epimer of the anthracycline antibiotic doxorubicin, with inversion of the 4'-hydroxyl group on the sugar moiety, and has been used alone or in combination with other cytotoxic agents in the treatment of a variety of malignancies (Young,

1989; Zuckerman et al., 1993). This drug is commonly used since it has an equivalent spectrum of antitumor action as doxorubicin but with less systemic and cardiac toxicity (El-Mahdy Sayed Othman, 2000).

The mechanism of anti-tumour action for EPI has not been completely elucidated. Various studies have revealed that EPI enters the cells rapidly and is localised in nuclei and forms a complex with DNA by intercalation between DNA strands (Di marco, 1984). DNA replication and transcription have been shown to be inhibited by this intercalation (Lollini et al., 1989;

Skladonowski and Konopa, 1994). In addition, topoisomerase-II has also been shown to be inactivated by EPI (Robert and Gianni, 1993; Haldane et al., 1993).

In vitro studies showed that EPI possesses cytotoxicity at least equivalent to that of doxorubicin against a variety of animal and human tumor cell lines including those derived from breast, liver, lung, gastric, colorectal, squamous cell, cervical, bladder, ovarian carcinomas, neuroblastoma and leukaemia (Bagnara et al., 1987; Zhang et al., 1992; Bartkowiak et al., 1992).

EPI is a cell cycle phase non-specific anthracycline, with maximal cytotoxic effects in the S and G_2 phases. Preliminary *in vitro* studies were carried out on HeLa cells. The first tests demonstrated that EPI and doxorubicin gave essentially the same inhibition of HeLa cell colony formation (Di marco et al., 1976). Similarly, EPI was as active as doxorubicin on mouse embryo fibroblast proliferation (Di marco et al., 1977), but was taken up in greater amount than doxorubicin by L1210 leukemia cells *in vitro* (Wilson et al., 1981).

There have been few studies about the effect of EPI on mitotic index of rapidly proliferating cells. In this study, we have therefore studied the effect at EPI, employed in the concentrations of 0.001 µg/ml, 0.01 µg/ml and 0.1 µg/ml for a period of 2 to 32 hours, on proliferation of transformed L-cells in culture which was investigated by measuring mitotic index in order to investigate the effectiveness of this drug in chemotherapy.

Material and methods

Chemical

EPI (4'-epidoxorubicin), an anthracycline antibiotic, 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 $^1C_4$ conformation, has an equatorial orientation (Plosker and Faulds, 1993).

Cell line

The cells used in this study were derived from mouse fibroblast by *in vitro* malign transformation (Earle,
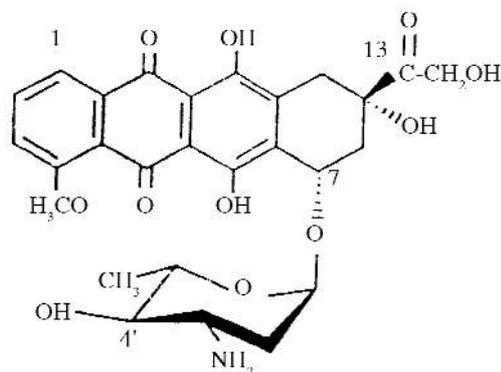

Figure 1: Structural formulae of EPI.

1943). Transformed L-cells obtained from mouse subcutaneous connective tissue in 1943. They were supplied by Dr. P.P. Dendy of Department of Radiotherapeutics, Cambridge University, in 1975. The cells were grown in Medium-199 (M-199, Gibco lab.) containing 10% foetal bovine serum (FBS, Gibco lab.), 100 µg/ml streptomycin and 100 IU/ml penicillin, and were passaged twice a week in appropriate number of 25 cm² flasks and the volume of the complete medium in each flask was completely to 12 ml. Cells were removed from the surface of culture flasks by addition of 0.25% trypsine (Gibco lab.) and centrifuged for 3 minutes at 1500 cycle/min. Following the addition of M-199 on the cell precipitate, the cells became ready for the experiment. Cell doubling time (Tc) of L-cells was 22.8 hours (Özcan and Rıdvanoğulları, 1996). L-cells were cultured on the cover-slips as 3.10^4 cells/ml in petri dishes and incubated for 24 hours with 95% air and 5% CO_2 containing medium at 37°C with pH 7.2 in a dessicator. At the end of this incubation medium was removed, replaced with medium containing EPI concentrations.

Drug application

Epirubicin (Farmorubicin, Carlo Erba) was dissolved immediatedly before use in sterile medium (M-199) to give the required concentration. We used 0.001 µg/ml, 0.01 µg/ml and 0.1 µg/ml concentrations of EPI. Cells were treated with these doses for 2, 4, 8, 16 and 32 hours.

Mitotic index 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
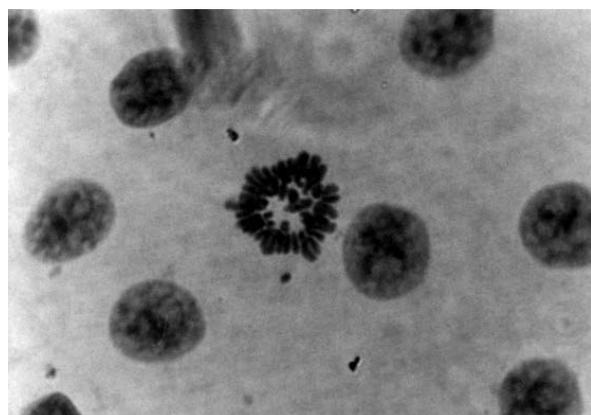

Figure 2: Mitosis in L-cells under the light microscope (3.3x100).

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 (Figure 2). At least three thousands cells were examined from each slide for mitotic index.

Statistical analysis

Mitotic index values which obtained from experiments were calculated to evaluate the statistical analysis. The differences between the percentage distrubition of M phase of the various treatment groups and control were compared by the Student-t test (n=25).

Results

The effect of EPI on mitotic index of L-cells in culture was investigated. EPI concentrations of 0.001 µg/ml, 0.01 µg/ml and 0.1 µg/ml were applied to the cells for time periods of 2, 4, 8, 16 and 32 hours. In this study, EPI diminished the mitotic index of L-cells with increasing both treatment time and drug concentration compared to controls (untreated group). From the value of 2 hours treatment, we saw that all EPI concentrations had a rapid effect. In subsequent hours, this effect seemed to continue. The values of mitotic index reached a minimum at EPI concentration of 0.1 µg/ml with increasing drug concentration. Table 1 reveals that treatments of EPI decreased the percentage of the cells at M phase. With increasing time the differences among the effects of various drug concentrations tended to be lower being very small at 2 to 8 hours applications. The inhibition of mitosis was higher in 16 and 32 hours applications than those in 2, 4 and 8 hours EPI applications in Table 1 especially with EPI concentration of 0.1 µg/ml. However, in the treatment of 0.1 µg/ml concentration, mitotic inhibition reached a maximum at 32 hours application. The values of mitotic index of the cells treated with EPI for 32 hours showed that mitotic index decreased as drug concentrations were increased.

Table 1: Mitotic index values in cultures of L-cells treated with various concentrations of EPI, given in mean ± Standard deviation (SD).

EPI concentrations	Mitotic index (%)				
	2 hours	4 hours	8 hours	16 hours	32 hours
Control	1.44 ± 0.12 [SD]	1.93 ± 0.14	3.04 ± 0.08	3.39 ± 0.15	3.84 ± 0.21
0.001 µg/ml	1.35 ± 0.09 [a]	1.80 ± 0.10 [a]	2.72 ± 0.07 [a]	2.96 ± 0.04 [b]	3.10 ± 0.30 [b]
0.01 µg/ml	1.29 ± 0.11 [a]	1.79 ± 0.05 [b]	2.61 ± 0.06 [b]	2.70 ± 0.13 [b]	2.99 ± 0.16 [b]
0.1 µg/ml	0.94 ± 0.02 [c]	1.04 ± 0.01 [c]	1.77 ± 0.09 [c]	1.85 ± 0.08 [c]	1.02 ± 0.22 [c]

[a]: $p < 0.05$, [b]: $p < 0.01$, [c]: $p < 0.001$

EPI significantly decreased the mitotic index in cultures of L-cells. The results show that EPI decreased the mitotic index at significant level $p < 0.05$ - $p < 0.01$ for lower drug concentrations 0.01 µg/ml and 0.001 µg/ml, at highly significant level $p < 0.001$ for 0.1 µg/ml drug concentration when compared with the control.

In addition, the reductions in mitosis of the cells following different treatment times (2, 4, 8, 16 and 32 hours) with 0.1 µg/ml EPI concentration were statistically significant ($p < 0.001$) from each other. However, this level of significance for the different treatment times was not observed with 0.01 µg/ml and 0.001 µg/ml concentrations of EPI, respectively.

Discussion

Anthracycline antibiotics have been used extensively in the treatment of wide variety of malignancies, and are a standard component of many combination chemotherapy regimens. EPI has been used alone or in combination with other antineoplastic agents in the treatment of a broad range of neoplasms. Studies in understanding the mechanism of EPI have suggested that EPI forms a complex with DNA by intercalation between DNA strands, thus inhibiting DNA replication and transcription (Özcan et al., 1997; Stewart et al., 1997), and it increases DNA strand brekage (Cantoni et al., 1990).

EPI induced differentiation of human neuroblastoma cells *in vitro*, possibly related to a reduction in the growth of surviving cells, thus allowing activation of intrinsic differentiation mechanisms. Following culture of human neuroblastoma cell lines with EPI 10 or 100 nmol/L for 24 hours, outgrowth of neurite processes was detectable 3 or 4 days after exposure, and maximal morphological differentiation was achieved after 5 or 6 days (Rocchi et al., 1987).

EPI has also been shown to be effective in inhibiting basement membrane degradation, a property deemed necessary to prevent development of metastases (Plosker and Faulds, 1993). In addition, 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 leukaemic cell lines in liquid culture (Bagnara et al., 1987).

Although, *in vitro* studies with antitumour agents, and with anthracyclines in particular, have not shown to predict the antitumour activity *in vivo* (Sinha and Politi, 1990; Nistico et al., 1999), in some studies, significant correlations have been detected between the *in vitro* activity of EPI and other anthracyclines against various tumour specimens, and therapeutic response (Bartkowiak et al., 1992; Plosker and Faulds, 1993).

Anthracyclines, including EPI, appear to result in maximal cell death in the S and G_2 phases of the cell cycle, but cytotoxic effects may occur in the G_1 and M phases at higher drug concentrations (Plosker and Faulds, 1993; Topçul et al., 2002). 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).

An important comparison between EPI and doxorubicin *in vitro* was carried out by Hill and Whelan (1982). The studies were performed on a wide range of murine and human cell lines: NIL8 (Syrian golden hamster cells); four human tumor lines (COLO-206 and LOVO, derived from colon carcinomas; SCC-T/G, derived from a squamous cell carcinoma from the tongue; CHP 100, derived from a neuroblastoma); L5178Y lymphoblastoid cell sub-lines. In the all cell lines tested, both drugs showed comparable cytotoxicity, which increased exponentially with drug concentration and with duration of exposure. Of high interest was the study carried out on NIL8 cells synchronized by mitotic selection and treated for 1 hour with the drug. Results showed that maximum cell kill was observed with both drug in the S phase, some kill during early G_2, but no kill in G_1 and M if low concentrations were used. Data from flow microfluorimetry analyses and monitoring of mitotic indices suggested population arrest in G_2 for doxorubicin (Casazza and Giuliani, 1984).

In the present study, treating L-cells with various concentrations of EPI for 2, 4, 8, 16 and 32 hours, decreased mitosis. The results showed that EPI diminished mitotic index of L-cells, depending upon time and applied concentrations. When compared to the control, this decrease was found statistically significant in each group ($p < 0.05$-$p < 0.001$). The values of mitotic index reached a minimum at EPI concentration of 0.1 µg/ml with increasing treatment time. Increased concentrations resulted in a decrease on the values of mitotic index, being statistically significant ($p < 0.001$). Briefly, EPI concentration of 0.1 µg/ml showed to possess relatively more effect on proliferation of L-cells. Thus, the results of our study

seem to be concordant with the above mentioned studies suggesting that cytotoxic effects of EPI might occur in the G_1 and M phases at higher drug concentration.

In our study, decreases in the mitotic index of cells with increasing both treatment time and EPI concentration have confirmed that EPI is an effective inhibitor of mitosis.

In conclusion, the results of this study declared the cell kinetics and cytotoxic effects of the anticancer drug, EPI, in treated cultures of L-cell line. Although EPI has less systemic and cardiac toxicity than doxorubicin and other anthracyclines with an equivalent spectrum of antitumor action, it still has cytotoxic effects.

Acknowledgment

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