

## The effects of epirubicin on proliferation and DNA synthesis of Ehrlich ascites carcinoma cells *in vitro* and *in vivo*

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In this study the effects of epirubicin (EPI), an antineoplastic drug, on cell proliferation, labelling index and mitotic index of Ehrlich ascites carcinoma (EAC) cells under *in vivo* and *in vitro* conditions were investigated. In the *in vitro* study, epirubicin was applied to EAC cells in doses of 1 µg/mL, 2 µg/mL, 4 µg/mL and 6 µg/mL. In the *in vivo* study, EPI 0.02 mg/g and EPI 0.04 mg/g were injected to mice intraperitoneally. These doses, applied both *in vivo* and *in vitro* studies, inhibited the proliferation of EAC cells as well as DNA synthesis and mitosis. It was found that this effect depended on increased amount of drug applied and time.

Key words: *in vitro*, *in vivo*, Ehrlich ascites, epirubicin, growth rate, labelling index, mitotic index.

### Introduction

In cancer therapy, besides surgery and radiotherapy, chemotherapy is also used frequently. In recent years, the consideration about antineoplastic drugs effects and information about cancer cell's kinetics have provided chemotherapeutic drugs to be used more comprehensively (SİPAHIOĞLU, 1993). The basic aim in the use of these drugs is to inhibit the proliferation of tumor cells or kill them without damaging the normal cells. Besides this, the drug's affective pattern, toxicity and side effects, which they will cause in relation to these criteria, are also evaluated (BAKEMEIER, 1981; EISENBRAND et al., 1984; NIAS, 1988; KAYAALP, 1989; BEGG, 1997). While having a toxic impact

on cancer cell, chemotherapeutic drugs may have a more or less toxic effect on normal cells. For this reason, in cancer therapy drugs causing the least damage to the normal cell are of preference for use (BUKOWSKI, 1996; DORR, 1996).

Epirubicin (EPI) (Fig. 1) is one of the anthracycline derivatives of antibiotics used frequently in chemotherapy currently (CASAZZA & GIULIANI, 1984; FORMELLI & POLLINI, 1989; YOUNG, 1989; PLOSKER & FAULDS, 1993; ROBERT, 1993; ROBERT & GIANNI, 1993). After intravenous administration to patients, EPI shows a wide dispersion in the tissues. As in the other anthracyclines, EPI depending on dose, causes bone marrow depression and as a result of which a decrease in the numbers of erythrocyte, leukocyte and thrombo-

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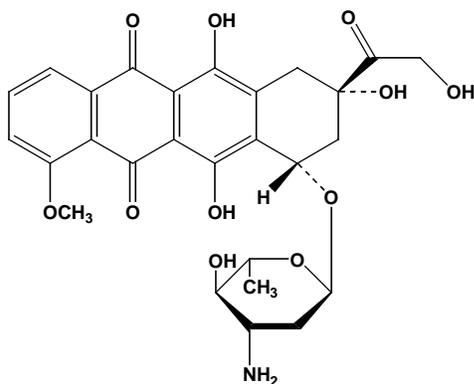


Fig. 1. The chemical structure of epirubicin.

cyte in blood occurs. Besides these, side effects such as nausea, vomiting, diarrhea, lack of appetite, cardiac toxicity are present. On the other hand, since EPI is tolerated better than doxorubicin, it is used in advanced breast cancer patients in high doses (OGAWA, 1994; HENDERSON, 2000). In clinic, EPI is administered to breast cancer patients in combination with cyclophosphamide and fluorouracil (CHOW & LOO, 2003). When the effect of epirubicin's mechanism is examined, it is observed that it can easily be bound to DNA. Cell culture experiments have shown that epirubicin is localized in nucleus and inhibits nucleic acid synthesis and mitosis (DI MARCO, 1984; YOUNG & WEENEN, 1984; SINHA & POLITI, 1990; PLOSKER & FAULDS, 1993).

Ehrlich ascites carcinoma (EAC) is one of the experimental breast tumor derived from spontaneous mouse adenocarcinoma. Similar to other tumors developing in body cavities, EAC cells fill the peritoneal cavity by rapid division. During this rapid increase, besides the tumor cells, accumulation of a fluid named ascitic fluid is also observed. With the growth of the tumor, the amount of the fluid increases and as a consequence, due to the pressure induced by both tumor cells and the ascitic fluid and also the tumor's damage to the organism, animal dies following 17-18 days of EAC transplantation (TANNOCK, 1969; ALTUN, 1996).

In previous studies, the effects of adriamycin, belonging to the same anthracycline group as epirubicin, on EAC and other types of tumor cells were investigated. It was established that these drugs had cytotoxic effects (ULAKOGLU, 2002). As these agents exert a cytotoxic effect on cells, they also affect plasma membrane transport mechanism. NAGAI et al. (2002) applied pirarubicin and doxorubicin to M 5076 ovarian sarcoma cells and Ehrlich ascites carcinoma cells and investigated the relation between cytotoxicity and transport system.

EAC cells multiply in two stages immediately after they are transplanted into the peritoneal cavity of mice. The first stage is the multiplication stage where the cell number increase exponentially and the other one is the plateau stage which follows the exponential one where the cell number almost stay stable (SKOG et al., 1990; LAZEBNIK et al., 1991; SIEMS et al., 1993; ALTUN, 1996). In the experiments, it was determined that EAC cells increase exponentially until 9<sup>th</sup> and 10<sup>th</sup> days after their transplantation and then enter the plateau stage (TANNOCK, 1969; ALTUN, 1996). TOPCUL et al. (1996) and AYDINER et al. (1997) gave EPI and tamoxifen in combination to EAC cells and examined both mitotic and labeling indexes of EAC cells and the effects of the drugs on cell cycle phases.

In this research work, the effect of four different epirubicin doses on the proliferation and DNA synthesis of EAC cells *in vitro* was investigated. The effects of EPI in doses 0.02 mg/mL and 0.04 mg/mL on the cell proliferation, labeling index and mitotic index of EAC cells *in vivo* were also examined.

**Material and methods**

*EAC cells*  
Hyperdiploid EAC cells (Robert Koch Institute-Berlin) were produced by transplantation from mouse to mouse routinely every twelve days. For the transplantation process tumor cells in the peritoneal cavity of the mouse were driven by a sterile injector and then by diluting with Hank's Balance Salt Solution (HBSS; Difco Lab.); a suspension of 10<sup>7</sup> cell/mL was thus obtained. Viability test was first made to the cells (PHILLIPS, 1973) and then 3 × 10<sup>6</sup> cells were implanted to each animal.

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##### *Preparation of drug density*

A stock solution was prepared by the addition of 5 mL sterile water to lyophilized 10 mg farmarubicin (epirubicin hydrochloride, Carlo Erba, Turkey). For the *in vitro* groups the dilution of stock solution was used: 1 μg/mL (EPI 1), 2 μg/mL (EPI 2), 4 μg/mL (EPI 4) and 6 μg/mL (EPI 6) concentrations were prepared. The stock solution was used in the *in vivo* groups.

##### *In vitro*

In order to carry EAC cells from *in vivo* conditions to *in vitro* medium, they were drawn with a sterile injector from the peritoneal cavity of mouse and by diluting with HBSS; 10<sup>7</sup> cells/mL concentration was thus obtained. Later, these cells were transferred to test tubes at a final concentration of 375,000 cell/mL. The test tubes contained MEM (Minimum Essential

Medium, Gibco) 2.5% Foetal calf serum (KAMINSKAS & NUSSEY, 1978; KRAKER et al., 1985) and 10 mM Hepes (Gibco). After the cells were brought to the *in vitro* method in this way, they were propagated at 37°C.

**Growth rate.** The cells in the *in vitro* medium were transferred to test tubes and incubated at 37°C. At the end of this period, the test tubes were separated into 5 groups. One of these groups was the control and the other four were experimental ones. To the experimental groups, epirubicin in EPI 1, EPI 2, EPI 4 and EPI 6 concentrations were added. 1, 24, 45 and 50 hours after treatment of the cells by the drug, trypan blue staining method survival test (PHILLIPS, 1973) was applied to the cells of both the control and the experimental groups. The number of surviving cells in each group was counted on the haemocytometer.

**Labeling index.** In order to determine the labeling index of EAC cells in the *in vitro* medium, they were treated as the cells of the growth rate experiments. 5  $\mu\text{Ci}/\text{mL}$   $^3\text{H-TdR}$  (TRA-120 Amersham) was added to the control and experimental tubes at 1, 24, 45 and 50 hours after drug application. The cells were kept in  $^3\text{H-TdR}$  for 30 minutes and then spread on gelatinized slides. The autoradiograms of these preparations were made and stained with Giemsa. By counting the labeled cells, the labeling index values of the cells were determined.

#### *In vivo*

Inbred male albino mice (*Mus musculus*) ( $n = 55$ ) of strain BALB/C, 20–28 g in weight were used in this study. All experimental animals were 2.5–3 months old and fed on their usual pellet and water ad. lib.

**Growth rate.** The mice were separated into three groups five days after  $3 \times 10^6$  EAC cells were transplanted into them. The first group, designed as control one, did not receive any drug. To the second group, five days after EAC cells transplantation, 0.02 mg/g EPI (EPI 0.02) was injected intraperitoneally. To the third group, again five days after transplantation, 0.04 mg/g EPI (EPI 0.04) was injected.

1, 4 and 7 days after EPI injection (6, 9 and 12 days after EAC inoculation, respectively) the mice of both control and experimental groups were sacrificed by servical dislocation, their peritonea were opened and after washing with HBSS all the tumor cells were collected. Surviving test was applied to the cells by trypan blue staining method (PHILLIPS, 1973) and total EAC cell number was determined by counting the surviving cells on the haemocytometer. In this way, total cell number of each mouse was determined.

**Labeling index.** In order to determine the labeling index, which renders the cell ratio of EAC cells synthesizing DNA, EAC cells were transplanted into mice and the drug was applied to them. 2  $\mu\text{Ci}/\text{g}$   $^3\text{H-TdR}$  was injected i.p. 1.5 hours before sacrifice on 1, 4 and 7 days to control and EPI 0.02 group mice. At the end of this period, the animals were killed by cervical dislocation. Smear preparations were prepared with EAC cells collected from the peritoneal cavity. Autoradiograms of these preparations were made with K2 gel

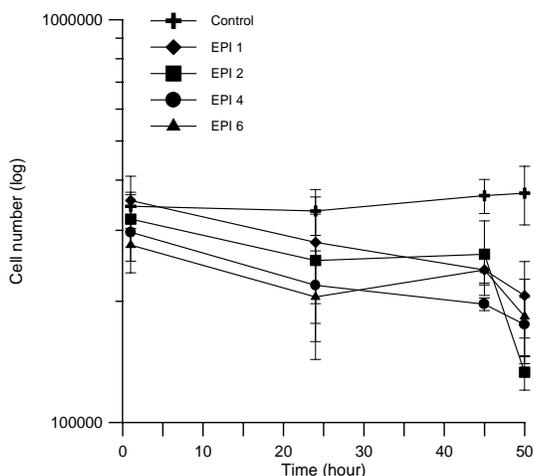


Fig. 2. The growth rate in the EAC cells ( $\pm$ SE).

emulsion (Ilford). After five days of exposure period, the slides were stained with Giemsa and by counting the number of labeled cells their labeling index values were estimated.

**Mitotic index.** Smear preparations were again made from cells on 1, 4 and 7 day of control and EPI 0.02 experimental group of mice. Following fixation, the cells were stained with Giemsa. By counting metaphase, anaphase and telophase stages of both control and experimental groups, the mitotic index values of the groups were estimated.

#### *Statistical analysis*

The values of growth rate, labeling index and mitotic index obtained in the both *in vitro* and *in vivo* experiments are given as arithmetic means of the groups and standard error of each mean. The significance of the variation among control and experimental groups was determined by Student *t*-test.

## Results

### *In vitro*

**Growth rate.** Cell numbers of both control and EAC cells *in vitro* at 1, 24, 45 and 50 hours after drug application in doses of EPI 1, EPI 2, EPI 4 and EPI 6 are shown in Figure 2. The examination of Figure 2 shows that until 50<sup>th</sup> hour a considerable increase in control group's cell number did not occur, but all the cells were surviving. The cell numbers of EPI 1, EPI 2, EPI 4 and EPI 6 experimental groups can be observed to decrease in comparison to the controls, after treatment with the drug. This decrease found in cell number with the drug treatment increased at 45 and 50 hours.

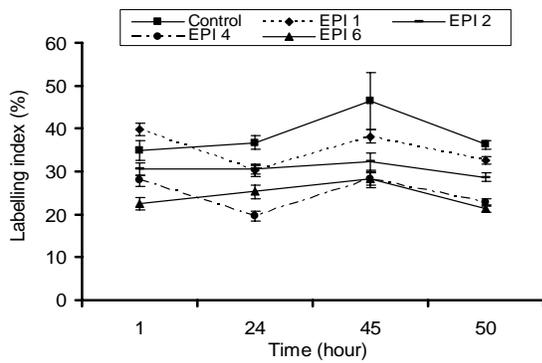


Fig. 3. Labeling index values of EAC cells ( $\pm$ SE).

When EAC cell numbers of the groups, treated with different doses of EPI, are compared with the control group, it is observed that a decrease in cell number occurred with the increase in dose concentration. The minimum amount of surviving cells was observed in dose EPI 4 group at 45<sup>th</sup> hour. This decrease was also found to be significant in EPI 1, EPI 4 and EPI 6 groups ( $P < 0.05$ ). Although the effective dose at 50<sup>th</sup> hour can be seen in EPI 2 group, it was found that a significant decrease ( $P < 0.05$ ) occurred in EPI 2 and EPI 4 groups.

**Labeling index.** The labeling index values of control and experimental groups are shown in Figure 3. When Figure 3 is examined, it is observed that labeling index values of EPI 1 and EPI 2 groups at 1 hour after drug application were not significant, even though they showed a little variance from the control groups. In the labeling index of EPI 4 and EPI 6 groups, a significant decrease ( $P < 0.05$ ,  $P < 0.001$ , respectively) in comparison to the control was found.

24 hour after epirubicin treatment, in the EPI 1 group's labeling index a significant decrease in comparison to the control  $P < 0.005$  and in EPI 2, EPI 4 and EPI 6 groups at  $P < 0.001$  level was observed.

When 45<sup>th</sup> hour is taken into consideration in view of labeling indexes, EPI 2, EPI 4 and EPI 6 groups exhibited a significant decrease ( $P < 0.05$ ) in comparison to the controls.

In the last group of epirubicin application at the 50<sup>th</sup> hour, the labeling index values of EPI 1 group at  $P < 0.005$  and in EPI 2, EPI 4 and EPI 6 groups at  $P < 0.001$  levels significant decreases in comparison to controls were observed.

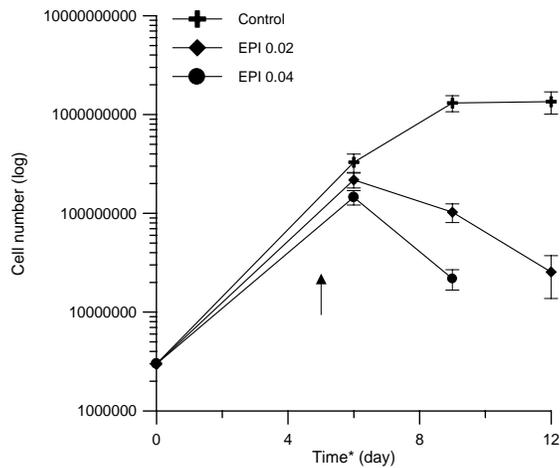


Fig. 4. The growth rate in the EAC cells ( $\pm$ SE). \* Time after the inoculation of EAC, arrow indicates drug administration.

#### *In vivo*

**Growth rate.** At the beginning, five days after the inoculation of  $3 \times 10^6$  EAC cells, EPI was administered in two different doses to mice. The cell numbers of control and experimental groups in 1, 4 and 7 days (6, 9 and 12 days after EAC inoculation, respectively) after drug administration are given in Figure 4.

Control group's EAC cell number (Fig. 4) reached to 328 541 666 on the 6<sup>th</sup> day after inoculation and to 1 308 226 000 on the 9<sup>th</sup> day. Tumor cell number of mice dissected on 12<sup>th</sup> day was 1 350 416 667. It was observed that multiplication of tumor cells obtained from the control group increased exponentially until the 9<sup>th</sup> day and from that day on they entered the plateau phase.

In the EPI 0.02 group (Fig. 4) it was observed that the cell number increased rapidly until the 1<sup>st</sup> day after drug injection and reached 217 784 690 cells, but after this day the cell number decreased. On the 4<sup>th</sup> day, while there were 102 570 833 cells, the cell number dropped to 25 492 500 on the 7<sup>th</sup> day.

In the EPI 0.04 group (Fig. 4) the EAC cell number was found to be 145 516 943 on the 1<sup>st</sup> day after drug injection which dropped to 21 830 000 on the 4<sup>th</sup> day. The mice of this group had diarrhea in the following days and could not survive until the 7<sup>th</sup> day.

In both EPI 0.02 and EPI 0.04 groups, after the 4<sup>th</sup> day (in increasing ratios) the intestines of the dissected mice were transparent, swollen and their spleens became smaller in size. During the

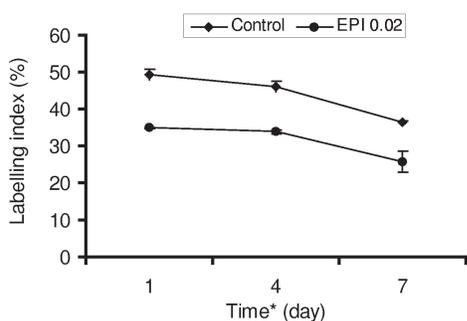


Fig. 5. Labeling index values of EAC cells ( $\pm$ SE). \* Time after the application of EPI.

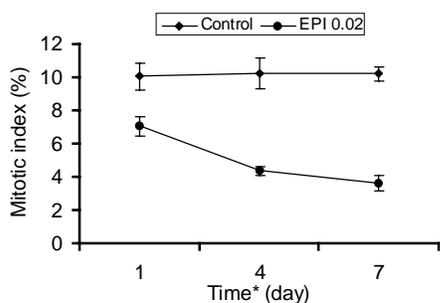


Fig. 6. Mitotic index values of EAC cells ( $\pm$ SE). \* Time after the application of EPI.

counting made under the microscope, besides the decrease in cell number of experimental groups after the 4<sup>th</sup> day many granules and particles, not present in the control group, were observed.

A significant decrease ( $P < 0.05$ ) in the cell number of EPI 0.04 group in comparison to the control group in the 1<sup>st</sup> day after drug administration was determined. On the 4<sup>th</sup> day, cell number of both experimental groups showed a significant difference in comparison to the control group (EPI 0.02:  $P < 0.005$ ; EPI 0.04:  $P < 0.05$ ). On the 7<sup>th</sup> day of the experiment, EPI 0.04 group's mice completely and EPI 0.02 mice partly died. Since n number decreased, a statistical evaluation could not be made.

**Labeling index.** Labeling index values for EAC cells obtained from control group's mice (Fig. 5) were found to be 49.214 on the 6<sup>th</sup> day, which decreased a little on the 9<sup>th</sup> and 12<sup>th</sup> days. It was observed that the labeling index values of EPI 0.02 group's cells (Fig. 5) were 34.896 in the 1<sup>st</sup> day after drug injection, which was smaller than that of the controls. This decrease continued during the

following days and on the 7<sup>th</sup> day the labeling index was found to be 25.754. When the labeling index values of EPI 0.02 and control groups were compared, it was observed that a significant difference ( $P < 0.001$ ) occurred during the period of investigation.

**Mitotic index.** The mitotic index values of control group's EAC cells (Fig. 6) were found to be 10.069 on the 6<sup>th</sup> day and remained at the same ratio also in the following days. During the 1<sup>st</sup> day of drug administration, the mitotic indices of EPI 0.02 group's EAC cells (Fig. 6) were found to be 7.051 which continued to decrease. On the 7<sup>th</sup> day, the mitotic index of EAC cells was found to be 3.621. When the mitotic index values of EPI 0.02 group and the control group were compared, it was observed that a significant difference ( $P < 0.01$ ) occurred during the period of investigation.

## Discussion

Many research works on the inhibition of cancer cell growth have been done for years. Besides various antineoplastic drugs, different plants and tissue extracts are also used in cancer therapy. CHARLES et. al. (1978) showed that when Ehrlich ascites cells were treated with normal mouse's skeletal muscle and liver tissue extracts, *in vivo* and *in vitro* tumor growth was inhibited.

We have determined that epirubicin doses used *in vitro* decreased both the growth rate and labeling index of EAC cells in comparison to the controls.

Studies were made on the effects of many drugs in anthracycline antibiotics groups to Ehrlich ascites tumor cells under *in vitro* conditions. When EAC cells were treated *in vivo* and *in vitro* with adriamycin, which is a member of this group, it was found that it exerted a cytotoxic effect to cells, and caused the death of the cells (CERCI & ULAKOGLU, 2000). SARASWATHI et al. (1997) treated Ehrlich ascites cells with adriamycin and echitamine chloride, both in combination and separately, and observed that they inhibited DNA, RNA and protein synthesis of the cells. In the *in vitro* experiments, it was observed that treatment of adriamycin in 2, 4 and 6  $\mu\text{g}/\text{mL}$  doses caused a cytotoxic effect dependent on time in EAC cells (ULAKOGLU, 2002).

BARTKOWIAK et. al. (1992) treated Chinese hamster's ovarian tumor cells with 0.1–20  $\mu\text{g}/\text{mL}$  epirubicin under *in vitro* conditions for 1 hour and observed their proliferation. They determined that a temporary cytostatic event occurred in the cells at 1  $\mu\text{g}/\text{mL}$  concentration, on the other hand the

cells died at 10 µg/mL. CHOW & LOO (2003) used epirubicin, cyclophosphamide and 5-fluorouracil in combination with cancer cell lines, MDA-MB-231 and MCF-7, and found that they inhibited the growth rate of the cells.

In this work, as a result of 0.02 mg/g EPI application to EAC inoculated mice, it was determined that the growth rate of tumor cells decreased rapidly. The impacts observed in the intestines of EPI 0.04 group mice are assumed to be due to drug's high dose and its side-effects. A parallelism in the results of labeling index and mitotic index values with growth rate can be observed only in EPI 0.02 group.

In the literature, several *in vivo* works where EPI is used in combination with other chemotherapeutic drugs can be found. Single and combined effects of EPI and tamoxifen to mitotic and labeling index values of EAC cells were investigated (TOPCUL et al., 1996). It was observed that after injection of 110 mg/60 kg EPI in third day following EAC inoculation, decrease occurred in mitotic and labeling index values of cells in 7<sup>th</sup> and 10<sup>th</sup> days. By giving EPI and tamoxifen in combination to EAC cells, AYDINER et al. (1997) examined the effects of the drugs on cell cycle phases. They observed that in the 5<sup>th</sup> and 10<sup>th</sup> days after drug application, while the cell number in G<sub>0</sub>+G<sub>1</sub> phases increased in comparison to controls, a decrease occurred in S and G<sub>2</sub>+M phases.

The effects of EPI and paclitaxel to mice with CH3/TIF having spontaneous breast adenocarcinoma were investigated (CIVIDALLI et al., 1998). In this work it was found that 9 mg/kg and 15 mg/kg EPI doses caused decreases in average tumor volume and cell number in S phase. By giving EPI (9 mg/kg), paclitaxel (45 mg/kg) and hyperthermia in combination to murine mammary adenocarcinoma cells, CIVIDALLI et al. (2000) analysed tumor growth delay. In this work, they observed that EPI + paclitaxel + hyperthermia treatment was the most effective one.

Besides EPI, the effects of some other chemotherapeutic drugs on EAC cells were also investigated. Bleomycine blocked the cell cycle of EAC cells and caused the accumulation of cells in G<sub>2</sub> phase (NAGATSU et al., 1972). Antitumor effect of adriamycin (0.2 mg/kg – 1.0 mg/kg) on solid EAC bearing mice was examined. They showed that adriamycin reduced the subacute toxicity of the mouse and increased the antitumor effect of the drug against solid EAC (MIMAKI et al. 1982). SHULTZE et al. (1985) examined the toxicity of vincristine, cis-platinum, cylophosphamide and 1-β-D-arabinofuranosylcytosine on EAC cells.

They showed that cis-platinum and cylophosphamide in high doses were toxic to EAC cells, which died before the controls. It was determined that a decrease occurred in EAC cell number after the administration of vincristine and 1-β-D-arabinofuranosylcytosine in low doses (SHULTZE et al., 1985). As in this work, ULAKOGLU (2002) observed that injection of 10 mg/g adriamycin four days after 3 × 10<sup>6</sup> EAC cell inoculation prolonged the life-span of mice 15 days in comparison to controls. These works are in accordance with our *in vivo* results.

Furthermore, the effects of the epidoxorubicin and epidoxoform on mouse mammary carcinoma model (Gollin-B mouse mammary tumor) were investigated. DERNEL et al. (2002) observed that epidoxoform (20 mg/kg) was more effective on tumor growth. Even though the use of higher doses than ours is mentioned in the literature, the toxic effects of drugs to animals are not mentioned.

Epirubicin is applied to cancer patients clinically. As a result of high dose epirubicin application, even though serious haematological toxicity is observed, it is still used in advanced breast carcinoma patients (HENDERSON, 2000).

According to the results obtained from this study, epirubicin doses applied to EAC cells derived from mouse breast adenocarcinoma under both *in vitro* and *in vivo* conditions inhibited cell proliferation and DNA synthesis. On the other hand, 0.04 mg/g EPI exhibited a toxic effect on cells from the fourth day on.

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