

Genotoxic Effect of Epirubicin in Mouse Bone Marrow *in vivo*

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Z. Naturforsch. **65c**, 211–217 (2010); received November 12/December 15, 2009

The genotoxic effect of epirubicin, a semisynthetic anthracycline antibiotic which has been used as an anticancer drug, was investigated *in vivo* on bone marrow cells of Swiss albino mice using the micronucleus test. To determine the incidence of micronuclei, mice were injected intraperitoneally with the drug at single doses of 4, 6, 8, and 10 mg/kg body weight. Then, bone marrow was sampled 18, 24, 36, and 48 h after the treatment. Polychromatic and normochromatic erythrocytes were examined for the presence of micronuclei. Epirubicin significantly increased the frequency of micronucleated polychromatic erythrocytes (MNPCEs) for all treatment periods compared with the negative control ($P < 0.001$). The frequency of MNPCEs increased with the dose, but at the highest dose used (which is considered to be quite toxic), the frequency of MNPCEs was rather lower. Epirubicin also decreased the ratio of polychromatic to normochromatic erythrocytes (PCE/NCE) for all sampling intervals, which is indicative of bone marrow cytotoxicity. It can be concluded from the present study that the anticancer drug epirubicin has genotoxic effects on mouse bone marrow cells.

Key words: Epirubicin, Micronucleus, Genotoxicity

Introduction

The anthracycline antibiotic epirubicin (4'-epi-doxorubicin) is an epimer of doxorubicin (adriamycin) with less cardiac and hematologic toxicity, but with similar mechanism of action and antitumour activity. Anthracycline antibiotics are among the most important antitumour agents and have been in clinical practice since the 1960's. The first generation anthracyclines, doxorubicin and daunorubicin, are produced by some *Streptomyces* species (Vermorken *et al.*, 1999). Although epirubicin differs from doxorubicin only in the orientation of the 4'-hydroxy group, this minor difference has important consequences on the spectrum of their activity (Minotti *et al.*, 2004). Doxorubicin is an essential component of treatment of human neoplasms, including a variety of solid tumours, whereas daunorubicin has been used primarily in acute leukemias. However, these compounds have severe adverse side effects, such as high cardiotoxicity, toxicity in healthy tissues or the development of resistance in tumour cells, the search for novel anthracyclines with more potent and less side effects has created numerous analogues (Muggia and Green, 1991; Minotti *et al.*, 2004). Among them, only few analogues,

including epirubicin, have reached the stage of clinical use.

Epirubicin is the 4'-epimer of doxorubicin and a semisynthetic derivative of daunorubicin, and is used widely as anticancer drug in Turkey and other countries. It has been approved by Food and Drug Administration (FDA, 2000). Epirubicin has been used extensively in the treatment of a wide range of cancers, including breast, ovarian, gastric, bladder, and colorectal carcinomas, lymphomas, leukemias, and multiple myelomas (Cersosimo and Hong, 1986; Muggia and Green, 1991; Coukell and Faulds, 1997; Vermorken *et al.*, 1999). It is used alone or in combination with other drugs.

Epirubicin is also an anthracycline antitumour antibiotic and cytotoxic agent. Anthracyclines can interfere with a number of biochemical and biological functions in cancer cells. However, the exact mechanisms of epirubicin's cytotoxic and/or antiproliferative properties have not been completely elucidated. Epirubicin is most active in the S and G₂ phases of the cell cycle, although, it shows activity in all phases of the cell cycle (Coukell and Faulds, 1997). The intercalation of epirubicin between DNA base pairs leads to the inhibition of DNA, RNA and protein synthesis (Piestrzeniewicz *et al.*, 2004; Wilmańska *et al.*, 2001). The other mech-

anism by which epirubicin effectively fights cancer is through the inhibition of topoisomerase II, an enzyme that normally functions during the process of DNA replication to produce transient double-stranded breaks in the replicating DNA to relieve torsional stress. Epirubicin inhibits the resealing of DNA breaks created by topoisomerase II, resulting in cytotoxic activity. Epirubicin also interferes with DNA unwinding or DNA strand separation and helicase activity. Other possible mechanisms are; generation of free radicals leading to DNA damage or lipid peroxidation, direct membrane effects, and DNA binding and alkylation (Minotti *et al.*, 2004; Nogrady and Weaner, 2005; Cantoni *et al.*, 1989; Tewey *et al.*, 1984).

The inductions of structural and numerical chromosomal aberrations were reported previously in cancer patients receiving epirubicin-containing chemotherapy (Pedersen-Bjergaard *et al.*, 1992). Previous studies have shown that epirubicin induces micronuclei in SCCVII murine carcinoma cells (Jeremic *et al.*, 1996), in the erythrocytes of incubated hen's eggs (Wolf *et al.*, 2008), and in the human lymphoblastoid TK6 cell line (Hastwell *et al.*, 2009), structural chromosome aberrations in cultured HeLa cells (Cantoni *et al.*, 1989), both structural and numerical chromosomal aberrations (CA) and sister chromatid exchanges (SCEs) in Chinese hamster cell line (Othman, 2000), and chromosomal aberrations and SCEs in peripheral blood lymphocyte cultures from women with breast cancer treated by epirubicin-containing regimen *in vitro* (Silva *et al.*, 2002), genotoxic effects in *Drosophila melanogaster* (Lehmann *et al.*, 2003), and clastogenic effects in rat bone marrow cells *in vivo* (Nersessian *et al.*, 1991).

Therefore, in the present study, we investigated the genotoxic effect of the anticancer drug epirubicin by the use of the micronucleus test *in vivo*. The micronucleus test on bone marrow cells of mice is an *in vivo* popular screening procedure for the detection of chemically induced structural and numerical chromosome aberrations (Maier and Schmid, 1976).

Materials and Methods

Chemicals

The drug epirubicin (Farmorubicin[®], Milan, Italy; CAS No. 56420-45-2) was obtained as a gift for experimental purposes from Farmitalia Carlo Erba Drug Company (Istanbul, Turkey). It

was dissolved in sterile distilled water at a concentration of 2 mg/ml shortly before being used. Fetal calf serum was purchased from Gibco Laboratories (Grand Island, NY, USA). Unless otherwise stated, all chemicals were purchased from Sigma Aldrich Chemical Co.

Animals

The male and female Swiss albino mice were obtained from Experimental Animal Centre of Uludag University (Bursa, Turkey). They were at the age of 6–8 weeks, housed in plastic cages with a bedding of wood shaving, and allowed to take food and tap water *ad libitum* throughout the experimental periods. This study was approved by the Ethical Committee on Animal Experiments of University of Uludag.

Doses and sampling times

The LD₅₀ value of epirubicin is 29.3 mg/kg for mice (Pfizer Ltd., 2006). Epirubicin was administered intraperitoneally (i.p.) to mice at single doses of 4, 6, 8, and 10 mg/kg body weight (b.w.). Four different sampling times (18, 24, 36, and 48 h) after injection were set for each dose. Negative and positive control animals received sterile distilled water and 200 mg/kg b.w. ethylmethane sulfonate (EMS), respectively. They were sampled once only 24 h after treatment.

Micronucleus test

Each dose group and negative control group contained 3 male and 3 female mice. The positive control group included only 4 mice (2 male and 2 female). A total of 106 animals were used for the whole experiment. The micronucleus test was performed according to the method of Adler (1984) with minor modifications as follows. At each sampling time, mice were sacrificed by cervical dislocation under diethyl ether anaesthesia. Both femora were removed from the freshly killed animals and then freed from muscles by the use of gauze and fingers. Bone marrow cells were flushed out with fetal calf serum from femur and the cell suspension was centrifuged at 100 × g for 5 min. The obtained cell pellet was then resuspended in a small volume of fetal calf serum and smeared on a clean glass slide. The preparations were dried overnight and then stained according to the following procedure: stained for 3 min in

undiluted May-Gruenwald solution [0.25% (w/v) May-Gruenwald's eosin methylene blue in methanol]; stained for 2 more min in May-Gruenwald solution diluted with distilled water (1:1); rinsed shortly in two changes of distilled water; stained for 20 min in Giemsa solution diluted with distilled water (1:19); rinsed in distilled water; air-dried; cleared in xylene for 5 min and mounted with a cover glass.

Microscopic evaluation

Slides were examined using a light microscope with a high power objective (100×). Micronucleus scoring was done in both polychromatic erythrocytes (PCEs) and normochromatic erythrocytes (NCEs) separately. 1000 PCEs per animal were analysed for the presence of micronuclei. The number of NCEs examined per 1000 PCEs was also recorded simultaneously, and the micronuclei in them were noted to estimate the percentage of micronucleated NCEs. Percentage PCE data were computed from the total red blood cells counted per animal. The ratio of polychromatic to normochromatic erythrocytes (PCE/NCE) was used to estimate the effect of the drug on the proliferative activity of bone marrow.

Statistical analyses

The statistical analyses were evaluated by the MINITAB release 12.1 for Windows programme (Minitab, 1998). The significance of differences between negative control and treatment groups in the percentage of PCEs, NCEs, micronucleated PCEs (MNPCEs) and micronucleated NCEs (MNNCEs), and the ratio of PCE to NCE and the ratio of MNPCE to 1000 PCE was statistically analysed by the Kruskal-Wallis (Kruskal and Wallis, 1952) and Tukey's multiple comparisons tests (Tukey, 1994).

Results

The results on the frequency of micronuclei in bone marrow erythrocytes of mice obtained with epirubicin are shown in Table I. Epirubicin significantly increased the frequency of MNPCEs for all treatment periods (18, 24, 36, and 48 h) compared with the negative control ($P < 0.001$) but it was increased significantly by epirubicin only at the 6-mg/kg b.w. dose for all treatment times ($P < 0.001$). The frequency of MNNCEs was increased

by epirubicin only at the 4-mg/kg b.w. dose for the 18-h sampling interval ($P < 0.05$). The maximum frequency of MNPCEs was observed at the 6-mg/kg b.w. dose of epirubicin in bone marrow and was 2.00% 36 h after treatment.

The ratio of PCE/NCE was significantly and dose-dependently decreased by epirubicin in the treated groups, and the highest decline was observed for the 10-mg/kg b.w. dose at all posttreatment times and was statistically significant ($P < 0.01$ and $P < 0.001$) when compared with the negative control (Table I).

The results of dose- and time-dependent frequencies of micronucleated PCEs are shown in Fig. 1. The percentage of MNPCE increased with time up to 36 h post treatment and peaked at 36 h for the 4-, 6- and 10-mg/kg b.w. dose and then declined sharply. The highest frequency of MNPCEs was seen at the 6-mg/kg b.w. dose after 36 h (Fig. 1).

Discussion

In the study presented here, we have investigated the genotoxicity of the anticancer drug epirubicin using the mouse bone marrow micronucleus test. It is very important to know the genotoxicity of an anticancer drug, because one of the potential long-term side effects of anticancer agents in non-tumour cells is their ability to induce cancer in another organ system years after the original cancer has been treated (Nogrady and Weaner, 2005). Epirubicin is an anthracycline anticancer drug, which interferes with DNA replication by intercalating mechanisms, and categorized as topoisomerase inhibitor (Nogrady and Weaner, 2005; Tewey *et al.*, 1984). Topoisomerases modify the topology of DNA without altering the deoxynucleotide structure and sequence. They can cause transient single-strand (topoisomerase I) or double-strand (topoisomerase II) DNA breaks that are resealed after changing the twisting status of the double helix. Topoisomerase II inhibitors cause DNA damage via inhibition of the ligase activity of topoisomerase II. Topoisomerase II inhibitors may produce DNA damage sufficient to induce secondary malignancies (Pedersen-Bjergaard *et al.*, 1992). The therapy-related acute promyelocytic leukemia (tAPL) occurs after a primary neoplasm treated in particular with topoisomerase II-targeted drugs such as anthracyclines (Beaumont *et al.*, 2003; Mistry *et al.*, 2005).

Table I. Effect of a single i.p. dose of epirubicin on micronuclei production in mouse bone marrow cells.

Samp- ling time [h]	Dose (mg/kg b.w.)	N	% PCE	% NCE	% MNPCE	% MNNCE	PCE/NCE	MNPCE/ 1000 PCE
24 ^a	0	6	49.90 ± 5.01	50.82 ± 4.18	0.03 ± 0.03	0.00 ± 0.00	1.00 ± 0.19	0.73 ± 0.56
18	4	6	46.07 ± 6.64	52.96 ± 6.62	0.82 ± 0.41	0.11 ± 0.07*	0.89 ± 0.23	18.52 ± 9.17
	6	6	46.24 ± 8.03	52.51 ± 8.62	1.20 ± 0.62***	0.02 ± 0.04	0.91 ± 0.28	24.72 ± 10.19***
	8	6	46.93 ± 5.10	51.87 ± 5.48	1.15 ± 0.50***	0.03 ± 0.04	0.92 ± 0.20	24.19 ± 8.09***
	10	6	19.53 ± 16.08**	80.20 ± 16.33**	0.24 ± 0.24	0.01 ± 0.03	0.28 ± 0.25**	9.05 ± 7.09
24	4	6	46.70 ± 3.01	52.18 ± 2.71	1.36 ± 0.17***	0.05 ± 0.04	0.89 ± 0.10	29.59 ± 5.06**
	6	6	42.66 ± 8.41	55.84 ± 9.05	1.49 ± 0.64***	0.03 ± 0.03	0.79 ± 0.28	33.79 ± 9.44**
	8	6	38.38 ± 14.50	60.28 ± 15.06	1.30 ± 0.66***	0.03 ± 0.03	0.73 ± 0.53	33.17 ± 9.43**
	10	6	19.82 ± 17.29**	79.91 ± 17.44**	0.22 ± 0.17	0.02 ± 0.05	0.29 ± 0.28**	15.20 ± 11.59
36	4	6	46.74 ± 5.53	51.25 ± 5.72	1.91 ± 0.63***	0.06 ± 0.06	0.92 ± 0.22	41.17 ± 13.99**
	6	6	38.38 ± 13.99	59.57 ± 14.13	2.00 ± 1.01***	0.07 ± 0.12	0.74 ± 0.55	50.79 ± 10.49**
	8	6	26.59 ± 17.91	72.88 ± 18.24	0.74 ± 0.42	0.01 ± 0.02	0.47 ± 0.44	33.51 ± 16.67
	10	6	12.90 ± 7.73**	86.43 ± 8.34**	0.48 ± 0.39	0.00 ± 0.00	0.15 ± 0.10**	26.77 ± 16.23
48	4	6	28.79 ± 6.92	69.43 ± 7.00	1.44 ± 0.27***	0.10 ± 0.10	0.42 ± 0.14	52.85 ± 16.50*
	6	6	15.21 ± 3.31***	83.99 ± 3.25***	0.82 ± 0.39***	0.09 ± 0.04	0.17 ± 0.04***	57.70 ± 37.77*
	8	6	11.38 ± 9.76***	88.13 ± 10.00***	0.31 ± 0.27	0.07 ± 0.07	0.13 ± 0.11***	43.22 ± 24.54
	10	6	3.45 ± 1.60***	96.39 ± 1.74***	0.14 ± 0.11	0.00 ± 0.00	0.03 ± 0.01***	36.14 ± 33.19
24 ^b	200	4	25.55 ± 2.45	73.69 ± 2.57	0.70 ± 0.13	0.03 ± 0.03	0.34 ± 0.04	27.82 ± 3.73

Abbreviations: N, number of animals per group; PCE, polychromatic erythrocytes; NCE, normochromatic erythrocytes; MNPCE, micronucleated polychromatic erythrocytes; MNNCE, micronucleated normochromatic erythrocytes. 1000 PCEs were analysed per animal. All data are presented as mean ± standard deviation.

^a Sterile distilled water. ^b Ethylmethane sulfonate (EMS).

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, significant when compared with the negative control.

In the present study, we have demonstrated that the anticancer drug epirubicin increased the frequency of micronucleated PCEs, and decreased the ratio of PCE/NCE indicating its genotoxic and cytotoxic effects in mouse bone marrow *in vivo*. Severe depression in bone marrow cells was observed in the 10-mg/kg b.w. group. The results of our work agree with the previously reported micronucleus induction by the anthracycline antibiotic epirubicin in murine cancer cells *in vitro* (Jeremic *et al.*, 1996), in the erythrocytes of incubated hen's eggs (Wolf *et al.*, 2008), and in the human lymphoblastoid TK6 cell line (Hastwell *et al.*, 2009). In addition, the anthracycline antibiotic doxorubicin has been reported to induce micronuclei, chromatid and chromosome breaks, and numerical chromosome aberrations *in vivo* and *in vitro* (Schmid, 1976; Maier and Schmid, 1976;

Nersessian *et al.*, 1991; Jeremic *et al.*, 1996; Dhanwan *et al.*, 2003; Venkatesh *et al.*, 2007).

Epirubicin is most active in the S and G₂ phases of the cell cycle, although, it exhibits activity in all phases of the cell cycle (Coukell and Faulds, 1997). In the present study, we observed high levels of micronucleated PCEs after epirubicin treatment, especially at the 36-h sampling time (Fig. 1). The highest frequency of MNPCEs was observed in the 6-mg/kg b.w. group after 36 h of epirubicin treatment (Table I; Fig. 1). After reaching a peak, there was a decline in the frequency of MNPCEs. These findings are consistent with the results of a previous study, in which the micronucleus frequency initially increased with culture time in murine tumour cells for epirubicin and doxorubicin at all concentrations, but after reaching peak levels it decreased (Jeremic *et al.*, 1996). Also, a similar

result has recently been reported by Venkatesh *et al.* (2007) who showed that the frequency of MNPCEs increased in a time-dependent manner up to 48 h, then declined; in this study, the mice were treated with different doses of doxorubicin, *i.e.* intraperitoneal injections of 0, 5, 10, or 15 mg/kg b.w. These researchers proposed that the reasons for the sudden decline in the MNPCE frequency 72 h after doxorubicin treatment may be the formation of micronuclei in erythroblasts during mitosis, the release of MNPCEs into the bloodstream before maturing into NCEs, dilution of micronuclei with successive cell division, removal of damaged cells, and dilution of the existing cells with the newly formed cells. Moreover, the degree of DNA strand breakages by epirubicin was not a linear function of exposure time; in fact, the rate of DNA strand-break induction decreased continuously with time (Cantoni *et al.*, 1989). Also, in an *in vitro* study with HeLa cells, DNA lesions caused by epirubicin were removed with a $t_{1/2}$ of about 1.7 h, whereas there was little repair of the DNA single-strand breaks produced by doxorubicin upon post-incubation for 5 h in a drug-free medium (Cantoni *et al.*, 1989).

Micronucleus induction can be the results of either chromosome breaking or whole chromosome loss due to non-disjunction. The micronu-

cleus test is able to recognize chemicals that disturb the function of the spindle, such as colchicine or related spindle poisons since whole chromosomes lagging in mitosis by losing the spindle may also form micronuclei (Adler, 1984). The micronucleus test is highly suitable for detecting chromosome loss due to partial impairment of the spindle apparatus (Schmid, 1976).

Epirubicin is classified into the topoisomerase inhibitors of cancer chemotherapeutic agents (interfere with DNA unwinding/repair). One of its toxicities is myelosuppression (Kasper *et al.*, 2005). In the present study, there was a decrease in the ratio of PCE/NCE in a dose- and time-dependent manner for all sampling times, especially at the 10 mg/kg b.w. dose, showing that epirubicin has effect on the proliferative activity of bone marrow. The results of myelotoxicity of epirubicin confirm the findings of Bagnara *et al.* (1987), that, *in vitro*, 0.058 $\mu\text{g/ml}$ epirubicin completely abolished the growth of all normal hemopoietic progenitor cells. Moreover, the anthracyclines cause myelotoxicity and reversible alopecia (Razis and Fountzilias, 2001). The micronucleus test permits a quick orientation about proliferation in the bone marrow and the composition. Therefore, the inhibition of proliferation is immediately recognized by lack of PCEs; severe destruction of cells mani-

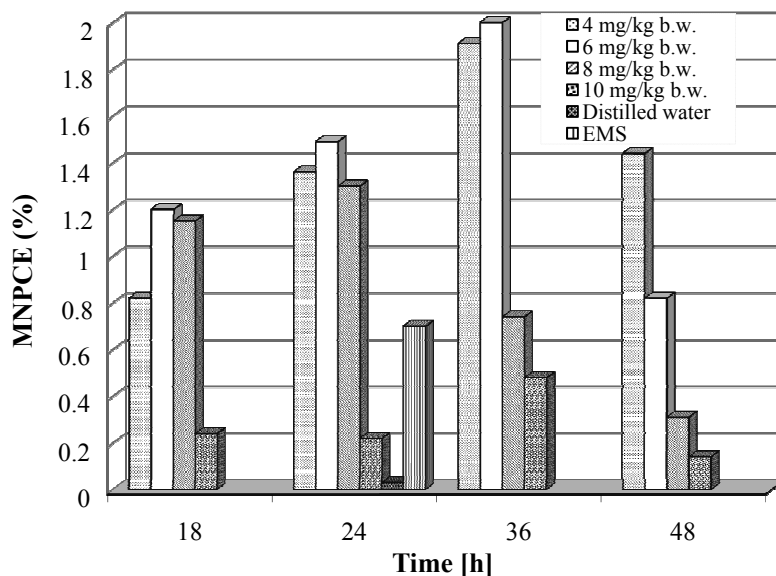


Fig. 1. Frequency of micronucleated polychromatic erythrocytes (MNPCEs) in mouse bone marrow treated with different doses of epirubicin. Ethylmethane sulfonate (EMS) was used as positive control.

fects itself by influx of peripheral blood (Schmid, 1976). Even after a single high dose, bone marrow depression is usually noticeable by a reduction of PCEs. Single treatment and multiple sampling times are best suited to avoid false negatives.

Ethylmethane sulfonate (EMS) is a well-known clastogen. Acentric chromosome fragments which are among chromosomal aberrations – the most probable if not the only candidates to generate micronuclei – are induced by clastogens, such as EMS (Van Hummelen *et al.*, 1992). In our experiments, it was used as positive control. This agent caused a significant increase in the frequency of micronucleated PCEs (Table I; Fig. 1).

In the present study, treatment of mice with different doses of epirubicin resulted in a dose- and time-dependent increase in the frequency of MNPCEs in mouse bone marrow cells. Micronuclei are formed from either chromatin fragments or whole chromosomes. In the work of Nersessian *et al.* (1991), chromosomal aberrations

induced by epirubicin and adriamycin in rat bone marrow cells consisted mainly of chromatid breaks (~ 90%). *In vitro* studies in different cell lines have shown that epirubicin increases both structural and numerical chromosome aberrations (Othman, 2000), single- and double-strand breaks in DNA (Cantoni *et al.*, 1989), and micronucleus frequency (Jeremic *et al.*, 1996). Moreover, in some cancer patients treated with epirubicin-containing chemotherapy, structural and numerical chromosome aberrations were found (Pedersen-Bjergaard *et al.*, 1992). Therefore, we propose that the micronuclei formed by epirubicin induction may arise from both acentric chromatid fragments and whole chromosomes, and epirubicin may be both clastogenic and aneugenic.

Acknowledgements

The authors are grateful to Farmitalia Carlo Erba Drug Company (Istanbul, Turkey) for the supply of the drug used in this study.

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