Cancer Chemother Pharmacol (1992) 30: 189–192

Cancer Chemotherapy and Pharmacology

Dose dependence of the cytokinetic and cytotoxic effects of epirubicin in vitro

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Received 17 July 1991/Accepted 2 March 1992

Summary. CHO cells were exposed in vitro for 1 h to concentrations of 0.1–20 μg/ml of the cytostatic drug epirubicin. Population growth, survival fractions, cell-cycle-phase distribution, and BrdU incorporation were analyzed. A fraction of the cells showed a transitory cytostatic reaction at 1 μg/ml, and >99% of the cells were killed at 10 μg/ml. The survival curve was biphasic with a steep slope at concentrations of up to 5 μg/ml. Approximately 0.1% of the cells were resistant to higher concentrations of epirubicin. Bivariate DNA/BrdU flow cytometry revealed that the sensitive cells were blocked and probably killed in the G_2M phase of the cell cycle.

Introduction

Epirubicin was introduced in cancer chemotherapy to reduce the cardiotoxic side effects of its parent drug doxorubicin [18]. Promising results have been reported in the treatment of different tumors such as lung [1, 13], breast [8, 9], and gastric cancers [14, 15], but failure of clinical chemotherapy has frequently been observed. Tumor-cell drug resistance is considered to be a major problem limiting the success of chemotherapy [4]. Since DNA is the major intracellular target of epirubicin, resistance to this drug may be due to DNA repair mechanisms [6]. Modifications of drug uptake, metabolism, and efflux can also influence tumor-cell survival, as has been found for several anthracyclines in vivo and in vitro [11, 19]. The effect of these drugs varies with the cell cycle, but the cell cycle is also altered by them [3]. Data on the resistance and cytokinetic response of cells may therefore be helpful in optimizing chemotherapy strategies. In the present study, the sensitivity and cell-cycle reactions of Chinese hamster ovary cells (CHO) to epirubicin were analyzed.

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Materials and methods

CHO cells were cultured in McCoy's 5a medium supplemented with 17% fetal calf serum at 37° C in water-saturated air containing 5% CO_2. Cells were seeded at a density of $5 \times 10^4 - 10^5$ cells in 25-cm^2 flasks. The experiments were carried out during the exponential growth phase at cell densities of approximately 2×10^6.

Epirubicin (Farmitalia, Freiburg, FRG) was diluted to 100 μg/ml in 0.9% NaCl. The cells were exposed to final drug concentrations of between 0.1 and 20 μg/ml ($1.84 \times 10^{-7} - 3.68 \times 10^{-5}$ M). After 1 h incubation, the cells were washed with phosphate-buffered saline (PBS).

Population growth was determined by counting the cells with a Coulter Counter immediately after drug exposure and on 4 subsequent days. To ensure maximal growth during that period, the medium was changed daily. The survival fractions were determined by seeding the drug-exposed cells into fresh and prewarmed medium. After colony formation (≥32 cells/colony), the cells were fixed and stained with 70% isopropanol containing 1% methyl violet.

For cell-cycle studies, the cultures were incubated with fresh medium for up to 24 h after drug exposure. At 2-h intervals, bromodeoxyuridine (BrdU)/deoxycytidine (Sigma, Deisenhofen, FRG) was added for 1 h to a final concentration of 150 μM. The cells were then trypsinized, fixed in 70% ethanol, and stored at –20° C. The fixed samples were sedimented for anti-BrdU antibody labeling according to standard techniques [10]. To denature native DNA to single-stranded DNA, which is essential for primary antibody attachment, 1 ml 1 *N* HCl was added to the cell pellet for 10 min at room temperature (RT). The acid was neutralized by the addition of 10 ml 0.1 M $Na_2B_4O_7$ and two washes with PBS.

Anti-BrdU monoclonal antibody (Partec, Münster, FRG) and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Dianova, Hamburg, FRG) were diluted 1 : 10 (v/v) with PBS. These solutions were stored at –20° C. The denatured cells were resuspended in 0.1 ml PBS containing 10 μl anti-BrdU solution and the suspension was incubated for 1 h at RT. The sample was again washed, resuspended in 0.1 ml PBS containing 10 μl FITC-conjugated goat anti-mouse IgG solution, and incubated for 1 h at RT in a dark environment. After being washed with PBS, the cells were stained with 2 ml of a DNA-specific dye solution containing 10 mg ethidium bromide/l (Serva, Heidelberg, FRG), 4 mg mithramycin/l (Serva), and 500 mg $MgCl_2$/l for at least 30 min at RT [20].

DNA and BrdU contents were measured using a PAS II flow cytometer (Partec, Münster, FRG). A software package (Phoenix Flow Systems, San Diego, Calif.) was used for the analysis of cell-cycle-phase distribu-

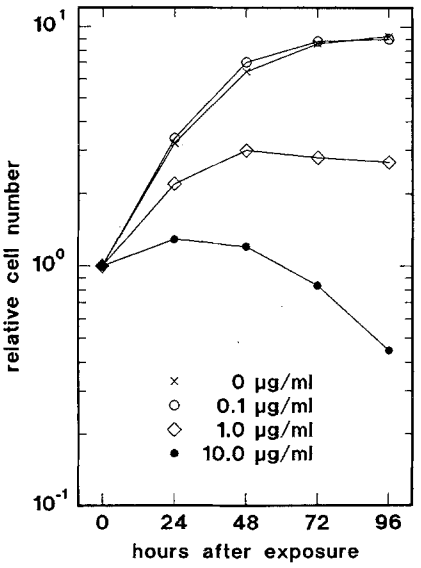

Fig. 1. Effect of epirubicin on CHO growth curves. Cells were exposed to the indicated drug concentrations for 1 h during their exponential growth phase and were refed daily to ensure maximal growth. Data represent mean values for the relative population size (cell number at t_0 = 1.0) in 3 sets of experiments. Coefficients of variation for the normalized data were <5%

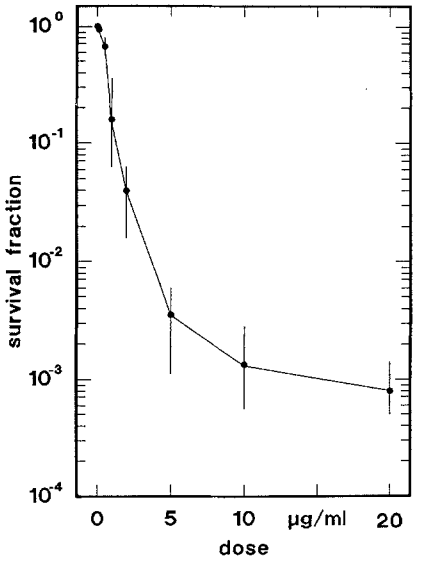

Fig. 2. Effect of epirubicin on CHO survival fractions. Cells were exposed for 1 h to drug concentrations of 0.1–20 µg/ml during their exponential growth phase. They were trypsinized and seeded at appropriate densities to yield 10–100 colonies of at least 32 cells. Data represent mean values and ranges for normalized data (controls = 1.0) in 3 sets of experiments

tion. The filter combination used for excitation included a BG 12 blue-green-band pass filter and a TK 500 dichroic mirror. The emitted light was split by a TK 560 dichroic mirror. An RG 590 long-pass filter was placed in front of the DNA photomultiplier, and an IF 510 interference filter combined with an OG 510 long-pass filter for compensation of side-band transmission was used for FITC analysis.

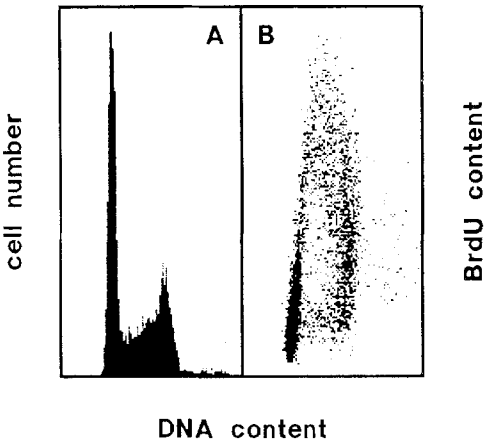

Fig. 3 A, B. Cell-cycle-phase distribution of exponentially growing, undisturbed CHO cells. **A** relative DNA content vs cell counts. The first peak represents diploid cells in the G_0/G_1 phase and the second, much smaller one represents cells in the G_2M phase. S-phase cells are characterized by an intermediate DNA content, which is displayed in channels between these two peaks. Bivariate flow cytometry enables synchronous acquisition of DNA content on the *x*-axis and BrdU content on the *y*-axis, with cell counts lying on the *z*-axis. **B** The horseshoe-shaped distribution shown in the scattergram indicates BrdU incorporation in S-phase cells exclusively. Integration of the respective areas enables the quantification of cells in the different cell-cycle phases. Only a minimal proportion of the cells with S-phase-equivalent DNA content are BrdU-negative

Results

The growth curves calculated for CHO cells after exposure to epirubicin are shown in Fig. 1. The initial values for every curve were normalized to 1.0. No alteration was observed at a drug concentration of 0.1 µg/ml. A concentration of 1 µg/ml reduced the plateau-phase density to 30% of that of untreated cells. The largest toxic effect was found after incubation with 10 µg/ml; within 4 days, the population size was reduced to 5% of the control value and 44% of the number of inoculated cells, respectively.

The dose response of CHO cells to epirubicin is shown in Fig. 2. The normalized survival fractions (mean values and ranges of data; controls = 1.0) were plotted as a function of drug concentration. Again, no measurable effect was found after treatment with epirubicin at a concentration of 0.1 µg/ml. When the dose was increased to 5 µg/ml, the rate of survival decreased rapidly to 0.5% but then fell with a flattened slope. A resistant fraction comprising about 0.1% of the seeded cells remained clonogenic at doses of up to 20 µg/ml.

For analysis of the immediate cytokinetic effects of epirubicin, DNA content and BrdU incorporation were determined by flow cytometry. A typical histogram of exponentially growing, untreated CHO cells is shown in Fig. 3. Figure 4 shows the effect of an epirubicin dose of 10 µg/ml on the cell-cycle-phase distribution over a period of 24 h. Within 14 h after drug exposure, >99% of the cells were arrested in the G_2M phase. Cells with an S-phase-equivalent DNA content incorporated BrdU. Thus, an interruption of DNA replication, i.e., an S-phase block, can be excluded.

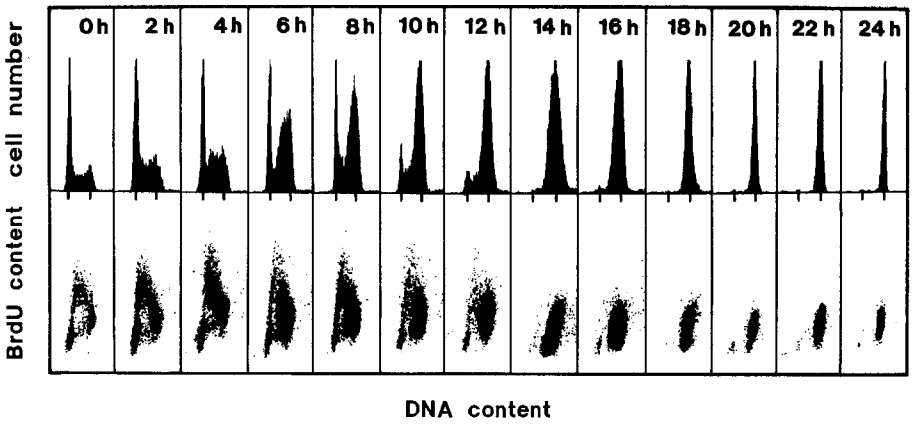

Fig. 4. Consecutive measurements of cell-cycle alterations induced by epirubicin. Exponentially growing cells were exposed to 10 μg/ml for 1 h and were then refed with drug-free medium. They were pulse-labeled (BrdU, 150 μM, 1 h) and harvested every 2 h thereafter. The *upper panel* shows DNA histograms and the *lower panel* DNA/BrdU scattergrams. Nearly all cells were arrested in the G_2M phase within 14 h after drug exposure. Incorporation of BrdU in all S-phase cells indicates continuous DNA replication

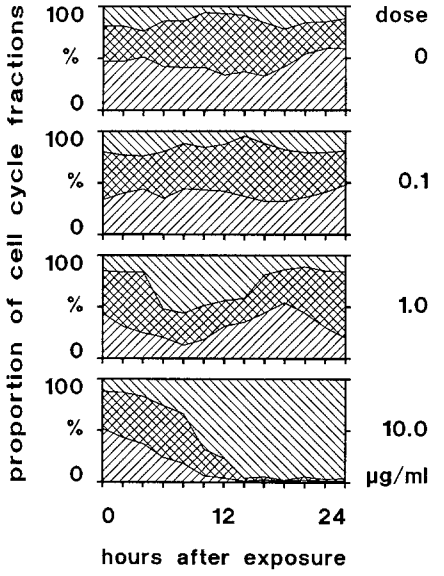

Fig. 5. Patterns of cell-cycle-phase fractions after 1 h exposure to epirubicin at the indicated concentrations. Samples were taken every 2 h. Calculations were based on the DNA histograms. The percentages of G_0/G_1 (*lower hatched area*), G_2M (*upper hatched area*) and S-phase (*cross-hatched area*) fractions are shown

The dose dependence of the cytokinetic effects of epirubicin is shown in Fig. 5. Comparable with the results obtained in the population-growth and clonogenicity experiments, no unambiguous effect of epirubicin was noted at 0.1 μg/ml. After exposure of the cells to 1 μg/ml, an increase to about 50% in the G_2M fraction was observed; however, after 18 h, the G_2M block was overcome. As indicated by the subsequent increase in the G_0/G_1 and S fractions and the corresponding decrease in the G_2M fraction, the cell cycle recommenced synchronously. In contrast to these observations, exposure of the cells to 10 μg/ml resulted in an irreversible G_2M block, which involved nearly 100% of the cells after 14 h.

Discussion

The results presented in this report correspond well to observations previously made using doxorubicin, the parent drug of epirubicin [2]. Both agents produce a biphasic dose-response curve for cell survival. For both drugs, the proportion of cells that were resistant to higher doses amounted to approximately 0.1%. CHO cells exposed to an epirubicin dose of 0.1 μg/ml reacted similarly to controls with respect to all parameters examined. In HeLa cells, a threshold value of 1 μg/ml has been established for the dose response to both doxorubicin and epirubicin, which was attributed to DNA repair [7]. In vivo, differences in DNA repair may be one reason for the unpredictability of the results of clinical chemotherapy [17].

After exposure of the cells to 1-μg/ml concentrations of epirubicin, population growth continued at a reduced speed, and the drug-resistant cells were characterized by a reduced plateau density. Studies on multidrug-resistant CHO cells have revealed changes in the membrane glycoprotein pattern [4]; this may not only influence the intracellular drug concentration but also affect the population size by altering cell-to-cell interactions.

Similar to the present findings for epirubicin, low doses of doxorubicin cause a reversible cell-cycle arrest in CHO cells [3], resulting in a partial synchronization of the cultures. The highest sensitivity to doxorubicin was found in synchronized, early-S-phase cells [12]. However, doxorubicin [16] and epirubicin caused G_2M arrest, regardless of the cell-cycle position at the time of drug exposure. Since the stringency of both the G_2M arrest and the cytotoxic effects increased with the epirubicin concentration, drug-dependent cell death is most likely to occur in the G_2M phase. In a rat tumor model, combination chemotherapy was most efficient when it was scheduled according to the recovery of synchronous proliferation after the administration of doxorubicin [5]. Our results provide cell-cycle data that can serve as a basis for similar in vivo experiments using epirubicin.

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