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Uptake, metabolism, and cytotoxicity of doxorubicin in human Ewing's sarcoma and rhabdomyosarcoma cells*

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Summary. *Uptake, metabolism, and cytotoxicity of doxorubicin (DOX) in human Ewing's sarcoma (ES) and rhabdomyosarcoma (RS) cells were examined. Cellular uptake of DOX was determined by liquid scintillation spectrometry, and intracellular metabolism was examined by high performance liquid chromatography. The cytotoxicity of DOX was assessed by two different methods: an extracellular matrix detachment assay (ECM-D) and ^{3}H-thymidine incorporation. The uptake of DOX by ES cells was 1.5–3.0 times greater than RS cells, even though both cell types achieved intracellular steady-state concentrations between 6–8 h. No significant intracellular metabolism (< 5%) was seen after 8-h incubations with the drug. The cytotoxic effects of DOX in both cell lines were concentration-dependent, with the RS cells being more sensitive. Measurment of ^{14}C-DOX appears to be a reliable method for quantitating intracellular DOX. In addition, the ECM-D and ^{3}H-thymidine assays used for assessing cytotoxicity produced similar results, showing that the ECM-D can be a reliable and easily performed test of cell death.*

Introduction

The anthracycline antibiotic, doxorubicin (DOX), is one of the most important anticancer drugs used in the treatment of a broad range of malignancies such as breast, ovarian, sarcomas, lymphomas, and acute leukemias [21]. The study of cellular pharmacodynamics and cytotoxicity of DOX is facilitated by the availability of reliable assays [3–5, 11, 12], the availability of malignant cell lines, and the fact that cells can be readily obtained from primary tumor sites of patients with cancer [8, 9, 17].

The development of cytotoxicity assays for use in predicting therapeutic response in patients with cancer has been the goal of many investigators [8–10, 14, 15, 19]. Even though cytotoxicity studies are still in the early stage of development, significant correlations with therapeutic response have been shown to occur with several types of malignancies [14, 19]. One criticism of current cytotoxicity assays is that cells are exposed to anticancer drugs for arbitrary times without consideration of in vivo pharmacokinetic events [1, 13, 19]. In a preliminary study, Andersson et al. demonstrated that cellular pharmacokinetics of daunorubicin correlates with cytotoxicity in an animal leukemia model [2]. The correlation between pharmacokinetics and cytotoxicity of DOX in human sarcoma cells has not been done.

In the present study, we examine whether uptake and drug-exposure times of DOX are related to cytotoxicity in two human sarcoma cell lines. In addition, a new method of assessing the cytotoxic effects of DOX was examined.

Materials and methods

Materials

Radiolabeled DOX hydrochloride (^{14}C-DOX specific activity = 29.7 μCi/mg) (NSC 123127) produced by the Stanford Research Institute (SRI), Palo Alto, Calif., was obtained from the National Cancer Institute. The purity of ^{14}C-DOX was documented by SRI using radioautography and in our laboratory by high performance liquid chromatography (HPLC). Before each experiment, the purity of stock solutions of ^{14}C-DOX and nonradiolabeled DOX was checked by HPLC. Drugs were stored at −20° C in the dark until the day of the experiment.

Extracellular matrix was prepared as previously described by Gospodarowicz et al. [7]. Tissue culture medium (DME, RPMI 1640) and phosphate-buffered saline were obtained from Grand Island Biological Co. (Grand Island, NY). Calf and fetal calf serum were obtained from Sterile System Co. (Utah), and tissue culture dishes were from Falcon Plastics. A 0.05% trypsin, 0.022 EDTA saline solution (STV) was from Difco, and ^{3}H-thymidine was purchased from New England Nuclear.

High-performance liquid chromatography (HPLC)

Identification of intracellular metabolism of DOX was accomplished with previously reported techniques [6]. To summarize, a DuPont model 850 gradient pump equipped with a Lichromsorb RP-18, 5 μM, 250 mm × 4.6 mm column, a Gilson GLO fluorometer, and a Spectra-Physics 4100 integrator were used in the detection of the fluorescent compounds. An isocratic system with a mobile phase of 50% acetonitrile, 40% water and 10% 0.1 M phosphoric acid was used at a flow of 1 ml/min.

Intracellular DOX metabolite doxorubicinol (DOXol) was identified by comparing the retention time of unknown peaks with those of DOXol. The percentage of metabolism was

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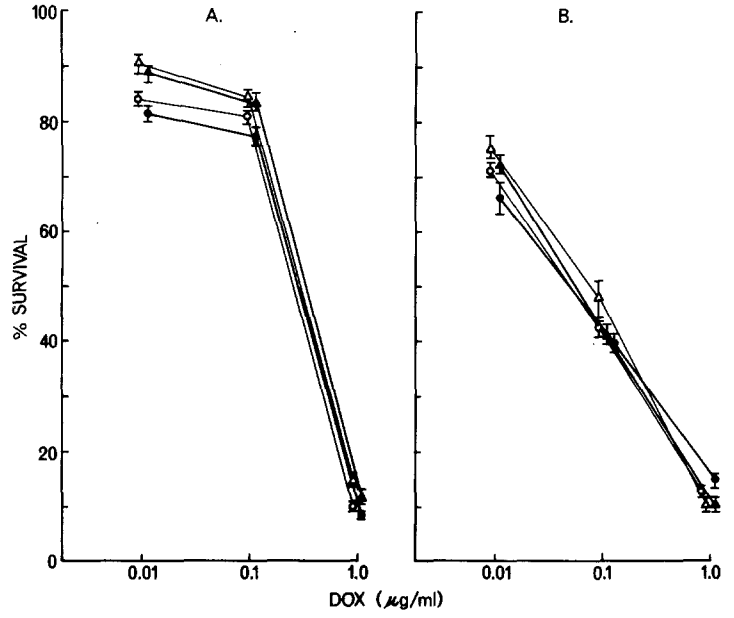

Fig. 1A and B. Cytotoxic effects of DOX in ES (**A**) and RS (**B**) when 0.1, 1.0, 5.0, and 10% fetal calf serum is present [(●———●), (○———○), (▲———▲), and (△———△), respectively]. Ranges are indicated by *bars*

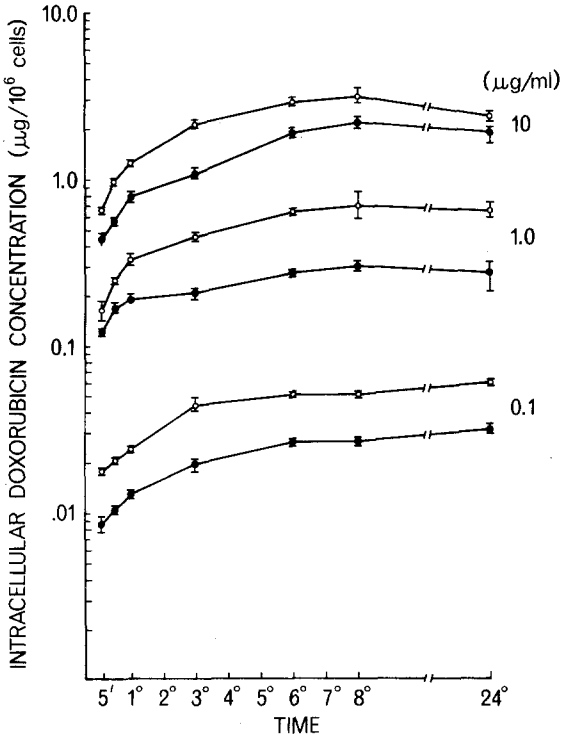

Fig. 2. Uptake of DOX by ES (○———○) and RS (●———●) cells at test concentrations of 0.1, 1.0, and 10.0 μg/ml. Each point is the average of three experiments. Ranges are indicated by *bars*

determined by dividing the area produced by the metabolite by the total area of parent plus metabolite.

Ewing's and rhabdomyosarcoma cells

The human Ewing's sarcoma (ES) and rhabdomyosarcoma (RS) cell lines were obtained from the Naval Bioscience Laboratory, Oakland, Calif. Both ES and RS cells were maintained in continuous semiattached culture at 37° C in an atmosphere of 10% CO_2 in air. Cells were subcultured every 3–4 days using DME H21 medium supplemented with 5% fetal calf serum.

Uptake of DOX

ES and RS cells were exposed to DOX in suspension culture to determine the cellular uptake of this compound. Radiolabeled ^{14}C-DOX was used as a tracer to measure intracellular DOX concentration per 10^6 cells [6]. The uptake of DOX was determined at three test concentrations, 0.1, 1.0, and 10.0 μg/ml. At the start of each experiment, the appropriate amount of DOX was added to triplicate 5-ml cell suspensions, which were then incubated at 37° C in 10% CO_2 in air. At several time points, triplicate cultures were rapidly cooled by placement in ice and then centrifuged at 2,000 rpm for 5 min. The amount of DOX in the supernate and in cell pellets were quantitated by liquid scintillation spectrometry. An additional flask was incubated with cells and DOX for examination of intracellular metabolism by HPLC.

Cytotoxicity of DOX
using the extracellular matrix detachment (ECM-D) assay

When maintained in plastic tissue flasks, the ES and RS cells grow as suspension or loosely attached aggregates. In an earlier study, we [18] demonstrated that ES cells form a cell monolayer composed of a firmly attached, highly flattened layer when seeded on an extracellular matrix (ECM). We also found this to be true for RS cells. Taking advantage of this system, we plate 1×10^6 cells in each 35-mm tissue culture dish previously coated with ECM in a medium containing 5% fetal calf serum (FCS). A 100% plating efficiency is usually observed. Both ES and RS cells were exposed to several different concentrations of DOX. After a 30-min, 3-h, or 6-h exposure to DOX, the cells were washed free of drug, and fresh medium containing 0.1% FCS was replenished. This low serum concentration has been observed to maintain 100% viability of the control plates (data not published) with only a slight (< 10%) increase in cell number over 5 days. In addition, cell survival after exposure to DOX was not altered by low serum concentrations (Fig. 1). Viability of cells exposed to

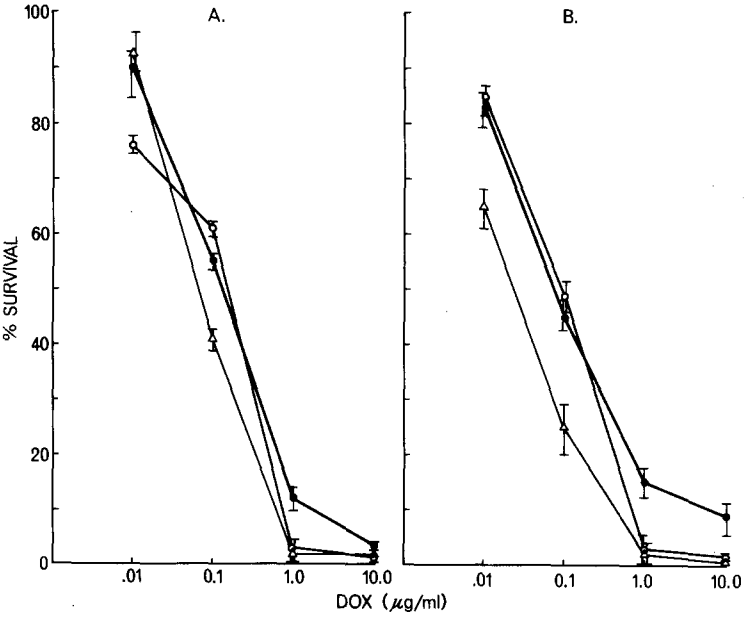

Fig. 3A and B. Cytotoxic effects of DOX in ES (**A**) and RS (**B**) cells after exposure times of 30 min (○———○), 3 h (●———●), and 6 h (△———△), as determined by the ECM-D assay. Each point is the average of three experiments. Ranges are indicated by *bars*

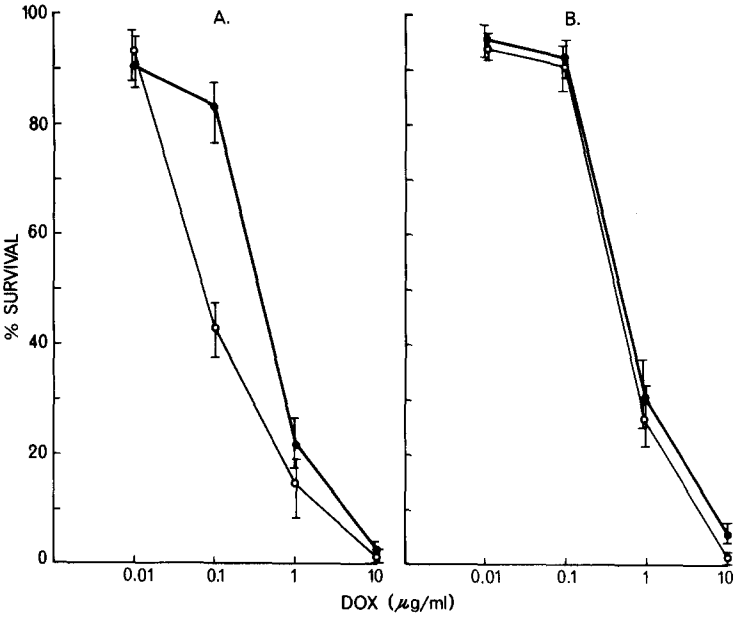

Fig. 4A and B. Cytotoxic effects of DOX in ES (**A**) and RS (**B**) cells after exposure times of 1 h (○———○) and 3 h (●———●) as determined by the ^{3}H-thymidine incorporation assay. Each point is the average of three experiments. Ranges are indicated by *bars*

DOX was determined by counting the number of cells still attached to the plate (viable cells) daily for 5 days. The nonviable cells (as documented by Trypan blue dye exclusion) detached and were removed by washing three times with PBS. The attached cells were removed by trypsinization and counted with a Coulter counter. By comparing the number of cells remaining attached in the DOX-treated plates with that of the total number of cells (detached plus attached cells), the percentage of survival was calculated.

Cytotoxicity of DOX using ^{3}H-thymidine-labeled method

A ^{3}H-thymidine incorporation assay was used to assess the cytotoxic effects of DOX. To summarize, triplicate samples of 1×10^6 cells/tissue culture tube were exposed to various concentrations of DOX for 1 and 3 h at 37° C. At the end of the incubation period, the cells were centrifuged at 2,000 rpm for 5 min. The medium was aspirated, and the cells were washed twice with PBS. Cells were resuspended in 2 ml of culture medium with 10% FCS and then plated on 35 mm tissue culture dishes. For radiolabeling, 5 μCi of ^{3}H-thymidine (specific activity, 2.0 M Ci/mmol) was added to each plate. The cells were incubated for 24 h and then rapidly cooled in a −20° C freezer for 10 min to prevent further incorporation. Cell samples were centrifuged and washed twice with PBS. The resulting cell pellets were completely dissolved in 1 ml of 1 N NaOH for 20 min at 37° C. After the cells were completely dissolved, 1 ml of 1 N HCl and 2 ml of cold 25% trichloroacetic acid were added to each tube. Each sample was shaken vigorously for 1 min and then allowed to stand at 4° C for 15 min. The samples were collected on filters and transferred to vials containing 10 ml of scintillation fluid. Radioactivity was determined with a Beckman LS-1215 scintillation

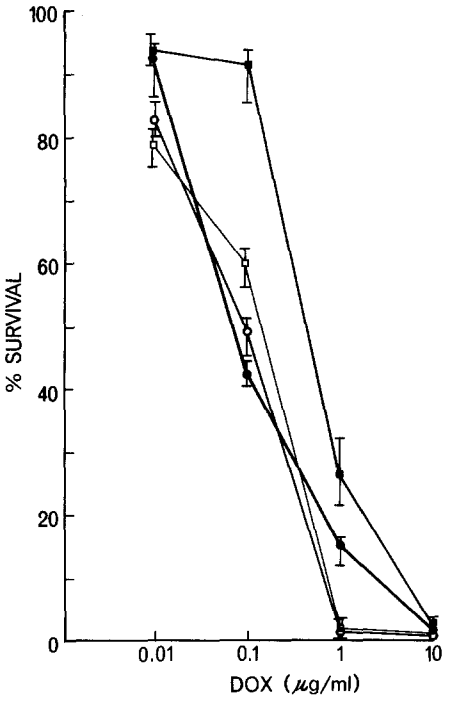

Fig. 5. Cytotoxic effects of DOX in ES (□———□) and RS (○———○) as determined by ECM-D assay, and cytotoxic effects of DOX in ES (■———■) and RS (●———●) as determined by ^{3}H-thymidine incorporation assay. Ranges are indicated by *bars*

counter. Percentage of cell survival was calculated by dividing the cpm of the drug-exposed cells by that of control cells.

Results

The uptake of DOX by ES and RS cells exposed to three different drug concentrations is shown in Fig. 2. The time to reach an intracellular steady-state level at all concentrations was 6–8 h after continuous drug exposure in both ES and RS cells. Even though the drug uptake pattern appears to be similar between the ES and RS cells, ES cells took up 1.5–3 times as much drug as the RS cells.

Because intracellular metabolism of the anthracyclines has been shown to occur with many types of cells [4, 6, 20], we examined ES and RS cells for the presence of DOX major metabolite, DOXol. We found that no significant (< 5%) intracellular metabolism was detected after 8 h of continuous drug exposure using the HPLC assay system in either ES or RS cells.

Using the ECM-D assay, the resulting cytotoxic effects at day 5 could be determined. Increasing cell death occurred when test concentrations were increased from 0.01 to 10.0 μg/ml. The percentages of cell survival after 30-min, 3-h, and 6-h incubations with DOX at day 5 are shown in Fig. 3A and B. Since the 30-min incubation is similar to the 3- and 6-h exposures with respect to cytotoxicity patterns, short incubation times can be used to assess the cytotoxic effects of DOX in these cells.

Since the ECM-D method for assessing cytotoxicity has not been previously reported, we also examined DOX cytotoxicity with a ^{3}H-thymidine incorporation assay. Since a 30-min exposure time was similar to the 3- and 6-h drug exposures in terms of cytotoxicity patterns for the ECM-D assay, only 1- and 3-h drug exposures were used for these experiments (Fig. 4A and B). Figure 5 shows the survival curves assessed by ECM-D and ^{3}H-thymidine assays. This figure shows that both the ^{3}H-thymidine and the ECM-D assay can be used to assess DOX cytotoxicity, but the percentages of cell survival at concentrations of 0.1 and 1 μg DOX are greater in the ^{3}H-thymidine assay system.

Discussion

In the present study, we examined human ES and RS cells to determine the uptake and cytotoxic effects of DOX. Our results show that the amount of DOX uptake by ES and RS cells in quite different even though the kinetic pattern is very similar. If achieving a critical intracellular concentration is necessary for cytotoxicity, then examining DOX uptake by patient cancer cells may be clinically important to obtain an accurate assessment of chemosensitivity.

Our results show that the cells that took up more DOX were less sensitive to its cytotoxic effects. Interestingly, the more rapidly dividing cells (12 h vs 16 h) are more sensitive to DOX. It is possible that both pharmacokinetic and cell-cycling time are equally important in determining the intracellular level of DOX required for cytotoxicity.

Assessing cell death or growth inhibition can be achieved by several methods. In the present study, we introduce the ECM-D assay. This assay is an easily performed measure of cell death, which correlates well with the ^{3}H-thymidine assay. Both ECM-D and ^{3}H-thymidine assays have an advantage over other methods in that a rapid assessment of cell viability can be obtained and can be used to study many different types of cells.

The simplicity of the ECM-D assay is an important consideration. It is true that preparing ECM costs more and is initially more time-consuming than purchasing ^{3}H-thymidine; however, only trypsinization and counting of the cells is required with the ECM-D. This takes much less time than processing a trichloroacetic acid precipitation for the ^{3}H-thymidine assay. Clearly, both assays are interchangable and can be used for assessing cytotoxicity.

The significance of these in vitro models for testing chemosensitivity in tumor cells can only be appreciated if they can be shown to correlate with clinical efficacy. Since cytotoxicity of DOX is concentration-dependent, determining a minimum effective dose necessary to achieve the cytotoxic effect may be possible. If an intracellular concentration can be determined, the correlation of in vitro and in vivo pharmacokinetic events may be more clinically relevant than present cytotoxicity studies examining exposure concentrations and time (C × T).

In summary, methods for examining the uptake, metabolism, and cytotoxic effects of DOX in human ES and RS cells are demonstrated. Further studies examining the interrelationship of cellular pharmacologic events (uptake, efflux, metabolism) and cytotoxic effects of DOX in these cells are in progress.

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