METABOLISM OF DOXORUBICIN IN LONG-TERM BONE MARROW CULTURES AND SR-4987 STROMAL ESTABLISHED CELL LINE

Laura Gribaldo[1], Paolo Catalani[1] and Marafante Erminio[2]

[1]*European Centre for the Validation of Alternative Methods (ECVAM)* and [2]*Environmental Toxicology and Human Health Unit, Environment Institute, JRC, Ispra, Italy*

SUMMARY

The metabolism of doxorubicin was studied in murine long-term bone marrow cultures (LTBMC) and in SR-4987 established stromal cells in comparison with primary cultures of murine and rat hepatocytes. The toxicity of metabolites was verified by testing their effects on the clonogenicity of granulo-macrophage progenitors. Metabolic activity was compared in subcellular fractions of SR-4987 cells and murine hepatocytes. Doxorubicin was transformed in long-term bone marrow cultures, SR-4987 cells and murine/rat hepatocytes to less toxic metabolites: 13-OH doxorubicin and a less polar metabolite which were non-toxic on granulo-macrophage progenitors. Among the hemopoietic compartments, stromal cells were responsible for the biotransformation of doxorubicin. The capability of the SR-4987 established stromal cell line to metabolize doxorubicin was higher than that of primary cultures of hepatocytes and bone marrow, and the highest activity was concentrated in the microsomes. These results suggest that *in vitro* models using primary cell cultures and established cell lines could be a useful tool for investigating the mechanisms underlying detoxification in the bone marrow stromal population.

KEY WORDS

doxorubicin, metabolism, cell culture, bone marrow, SR-4987 cell line

Author for correspondence:
Laura Gribaldo
ECVAM, J.R.C., 21020 Ispra, Italy
Fax +39 332 785336; E-mail: laura.gribaldo@jrc.it

INTRODUCTION

Bone marrow stromal cells constitute a micro-environment supporting the development of progenitor cells and releasing cytokines involved in the regulation of hemopoiesis /1/. This tissue is a specific target for the toxicity induced by antineoplastic agents such as busulfan /2/ or the myelotoxic solvent benzene /3/ and there is evidence indicating that it has some capability to metabolize and detoxify xenobiotics /4/ through drug metabolizing enzymes such as cytochrome P-450, although the specific activities are much lower than in the liver /5-7/.

Long-term bone marrow cultures (LTBMC) allow the proliferation and differentiation of hematopoietic cells to be maintained for many weeks /8/, and thus offer the opportunity to delineate *in vitro* mechanisms associated with xenobiotic toxicity toward the hemopoietic micro-environment /9/. Moreover they permit investigation of the metabolic activity of a primary culture over a prolonged time to verify the maintenance of its capability for drug biotransformation.

Immortalized stromal cell lines such as SR-4987 /10/ could be used to analyze the complex interaction between stem cells and stromal cells, and the metabolic competence of bone marrow stroma in more standardized conditions /11/.

Doxorubicin is an antineoplastic agent widely used to treat acute leukemia and solid tumors /12/. The plasma pharmacokinetics of doxorubicin and its metabolites have been studied in humans /13/, showing the presence of aglycone and polar metabolites, whereas little doxorubicinol and doxorubicin aglycone have been shown to be produced in *in vitro* cultured rat hepatocytes /14/. Therapeutic doses of this anthracycline produce toxic effects on bone marrow and peak plasma levels may reach 6-7 μM.

In the present study we investigated the metabolism of the antineoplastic agent doxorubicin in murine long-term bone marrow cultures and in the established stromal cell line SR-4987. The toxicity of the metabolites was evaluated by the agar clonogenic assay on the granulo-macrophage progenitors from long-term cultures. We compared the metabolic activity measured in SR-4987 cells and LTBMC with that determined in primary cultures from murine and rat hepatocytes. The production rate of doxorubicin metabolites was analyzed in

microsomes and cytosol from murine hepatocytes and SR-4987 subcellular fractions.

MATERIALS AND METHODS

Drugs

Doxorubicin (DXR) (Fluka, USA) and 13-OH-doxorubicin (13-OH) (kindly supplied from Pharmacia, Nerviano) were dissolved in bidistilled water to the concentration of 10 mg/ml. Working dilutions to the final concentrations were performed in culture medium.

Cells

Committed precursors for the agar clonogenic assay were obtained from long-term murine bone marrow cultures established under Dexter-type conditions /15/, re-fed weekly with Myelocult 5300 (Stemcell Technologies, Vancouver, BC, Canada), with the addition of 10^{-6} M hydrocortisone hemisuccinate, and maintained at 33°C in 5% CO_2.

The SR-4987 cells (obtained from Dr. A. Pessina, Institute of Microbiology, Milan) were subcultured weekly in 5A McCoy's medium (Gibco Life Technologies, Paisley, Scotland, UK) supplemented with L-glutamine (4 mM), penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% fetal calf serum (FCS; Gibco Life Technologies).

The hepatocytes for the primary cultures were isolated from male CD1 mice by the collagenase method /16/ with preperfusion and perfusion steps done *in situ*. The viability was always higher than 80% as assessed by Trypan Blue exclusion assay. The cells were resuspended in Williams medium (Gibco Life Technologies) supplemented with serum (FCS; Gibco Life Technologies) and seeded onto Nunc 24-well plates at a density of $7x10^4$ cells per well. Hepatocytes were allowed to attach at 37°C in 5% CO_2. After 3 h the supernatant was replaced with fresh medium, without serum, with addition of the drug.

Doxorubicin exposure

The layers of LTBMC and SR-4987 cells were washed and the supernatants replaced with complete medium containing doxorubicin

to a final concentration of 0.5 μg/ml. After 24, 48 or 72 hours of incubation at 37°C the supernatants were collected and analyzed. The primary cultures of hepatocytes were treated with 10-30 μg/ml of doxorubicin for 4 and 24 hours. The doses were previously tested (data not shown) to avoid any cytotoxic effect during the time of exposure.

Colony-forming unit granulocyte/macrophage (CFU-GM) assay

The proliferation of murine myeloid precursors was assayed in a soft agar system, according to the method of Bradley and Metcalf /17/. Briefly, murine bone marrow cells were cultured in 1 ml of 0.3% agar-Myelocult medium plus 20% FCS. Each culture received 80 U/ml of a standard Colony Stimulating Factor (CSF) obtained from cultures of WEHI-3B myelomonocyte leukemic cells (ECACC 86013003).

The effects of doxorubicin, 13-OH-doxorubicin and a non-identified metabolite (DXR-met) on GM-CFU proliferation were measured by adding 100 μl of the test chemical (twofold dilutions from 0.003 to 0.2 μg/ml) to each culture dish. The number of colonies (>50 cells) was scored with an inverted microscope after 7 days of incubation at 37°C in 5% CO_2. Three experiments in duplicate were carried out.

Preparation of subcellular fraction from mouse liver hepatocytes and SR-4987

Mouse liver was homogenized 1:4 (w/v) in 250 mM sucrose with 50 ml potter at 4°C. The suspension was divided into two ultra-centrifuge tubes. $8x10^8$ SR-4987 cells were trypsinized and resuspended in 20 ml bidistilled water, sonicated 10 times for 10 min (15 min break) and the solution was divided into ultracentrifuge tubes. 10 ml of a 500 mM sucrose solution were added to each tube. The centrifugation steps were the same for both biological samples: 16,300 *g* x 30 min to obtain a supernatant which was centrifuged at 100,000 *g* x 60 min to separate the microsomes in the pellet and cytosol as supernatant /18/. The determination of the amount of protein was carried out with the Biorad DC protein assay kit.

Cytochrome P-450 determination in mouse liver and SR-4987 microsomes

Microsomes were resuspended in phosphate buffer 0.1 M, pH 7.4 to give a final concentration of 1-1.5 mg/ml of protein. The material was mixed, divided into two portions, and placed in 1-ml glass cuvettes (10 mm path length). Baseline was recorded between 400 and 500 nm. The sample cuvette was saturated with 40 bubbles of CO. A few crystals (1-2 mg) of sodium dithionite ($Na_2S_2O_4$) were added to each cuvette which were then well shaken and placed in the spectrophotometer. Spectra were recorded until the 450 nm peak was at the maximum. Abs 490 nm was the reference point. Cytochrome P-450 content was determined as follows:

[(Abs 450-490)measured - (Abs 450-490)baseline]/0.091
= nmol cytochrome P-450/ml

Sample preparation

SPE Supelclean LC-18 (400 mg, 1 ml) columns were used to purify the cell culture medium samples in a Visiprep DL Supelco vacuum system (12 samples simultaneously). The columns were conditioned with 2 ml methanol and 2 ml water, loaded with 1 or 2 ml of sample twice, washed with 5 ml of water and dried. The compounds were eluted twice in 0.5 ml 60% acetonitrile/1% acetic acid. Finally the dry samples were dissolved in 10% acetonitrile/1% acetic acid and injected onto the column (50-200 µl).

HPLC system and chromatographic method

HPLC analysis of drug metabolites was performed using a Waters 600E pump equipped with a Rheodyne injector valve (Model 7725I) with a 200 µl loop and Waters 996 photodiode array detector set at 230 nm. Fluorescence detection was performed using a Varian 9070 spectrofluorimeter with an excitation wavelength of 480 nm and an emission wavelength of 560 nm. All data were managed by the Waters Millenium Chromatography Manager v. 2.1 software. The column was a Supelcosil LC-18, 5 µm, 250x4.6 mm with pre-column 20x4.6 mm. The eluent mixture was acetonitrile and 1% acetic acid, pH 4.3. The gradient mode was used for better characterization of the metabolites. The acetonitrile/acetic acid solution ratio was maintained at 10/90

(v/v) for 1.5 min and then raised linearly to 80/20 over 19 min and maintained for 2 min with a flow rate of 1.5 ml/min. The metabolites were isolated by fractionating injections of 1 ml samples with a Gilson 203 automatic collector.

RESULTS

Figure 1 shows the HPLC analysis of the supernatant from SR-4987 cell cultures, long-term bone marrow primary cultures and murine hepatocytes at 24 h after exposure to 0.5 μg/ml of doxorubicin (SR-4987 cells and LTBMC) and 10 μg/ml (murine hepatocytes).

13-OH-Doxorubicin (13-OH) and an unknown metabolite less polar than 13-OH were produced. It is suggested that this unknown metabolite might be the result of the demethylation of the 7-deoxy-aglycone, but its characterization awaits further analysis. Both 13-OH and the unidentified metabolite (DXR-met) were tested for their activity on GM-CFU proliferation (Fig. 2). It can be seen that 13-OH was less toxic than DXR with an IC_{30} of 0.05 μg/ml, whereas DXR-met did not exert any toxic activity at concentrations up to 0.2 μg/ml.

The histogram in Figure 3 compares the 24-hour production of 13-OH and DXR-met in bone marrow stromal cells, SR-4987 established cell line, rat and mouse hepatocytes. At 24 hours, rat and murine hepatocytes showed a similar activity to the stromal cells in producing 13-OH, whereas rat hepatocytes produced less DXR-met. The production of metabolites in the different hemopoietic compartments is shown in Figure 4. Bone marrow stromal cells were mainly responsible for biotransformation.

Table 1 shows the production rates of DXR metabolites in microsomes and cytosol from murine hepatocytes and SR-4987 cells. Microsomes from mouse hepatocytes transformed DXR mainly to DXR-aglycone (DXR-one) and 13-OH-aglycone (13-OH-one), whereas microsomes from SR-4987 cells produced DXR-met and DXR-one.

The cytosolic fraction showed a higher activity of aldo-keto reductases, producing 13-OH in both cell populations which was quickly transformed to 13-OH-one in cytosol from mouse hepatocytes and the 13-hydroxy-7-deoxyaglycone in SR-4987 cells. The data underline the lower rate of production of 13-OH (cytosol) and DXR-one (microsomes) in SR-4987 stromal cells whereas these cells showed a significantly higher rate of production of DXR-met.

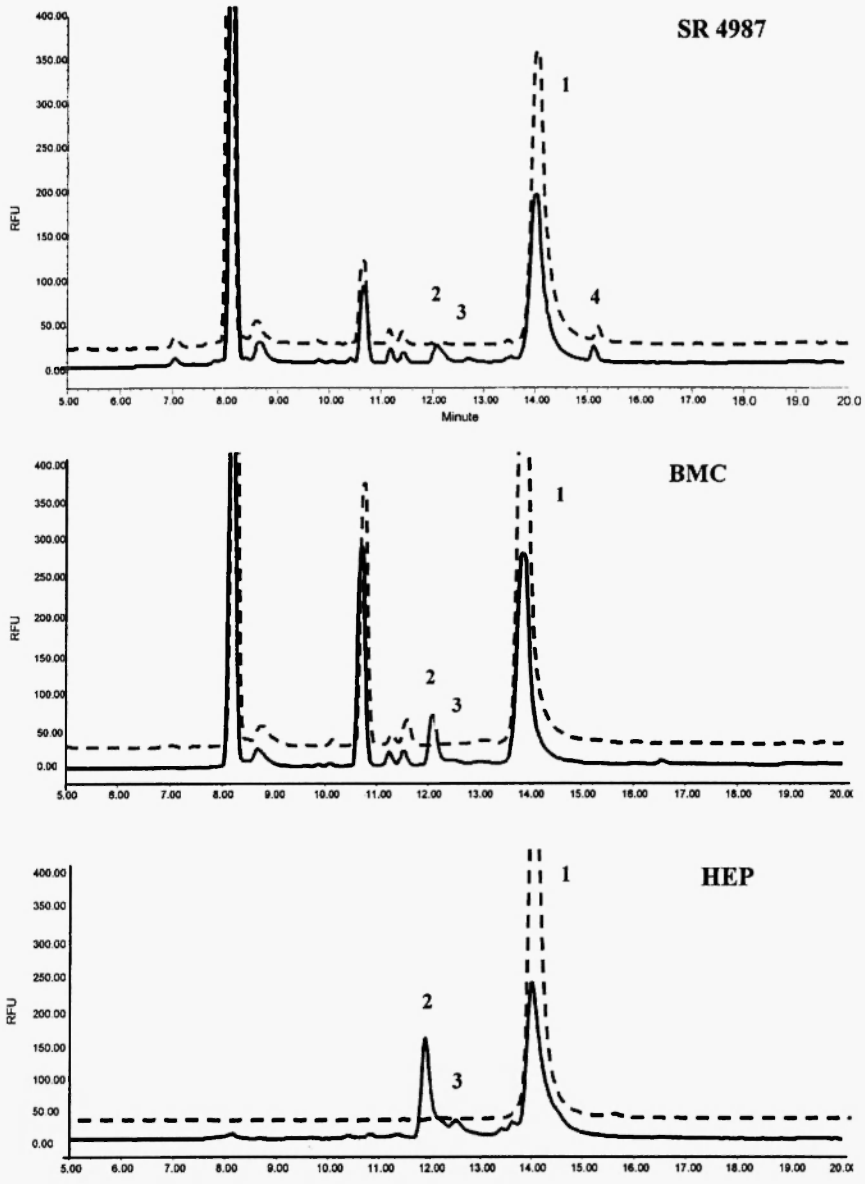

Fig. 1: HPLC analysis of the supernatant from SR-4987 cell cultures (SR-4987), long-term bone marrow cultures (BMC) and murine hepatocytes (Hep) at 24 hours after exposure to 0.5 μg/ml (SR-4987; bone marrow cultures) and 10 μg/ml (murine hepatocytes) of doxorubicin. - - - T = 0 h; — T = 24 h; 1 = doxorubicin (DXR); 2 = unidentified metabolite (DXR-met); 3 = 13-OH-doxorubicin (13-OH); 4 = doxorubicin aglycone (DXR-one).

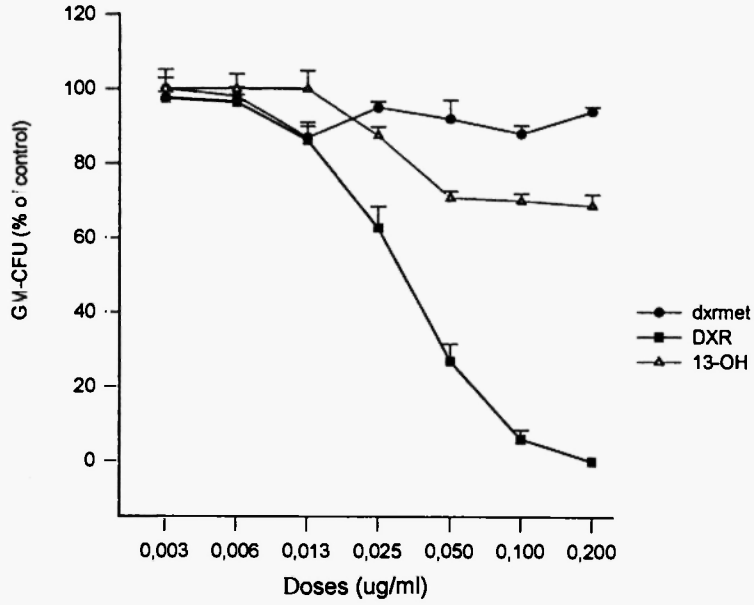

Fig. 2: Effect of doxorubicin (DXR), 13-OH-doxorubicin (13-OH) and unidentified metabolite (DXR-met) on GM-colony formation.

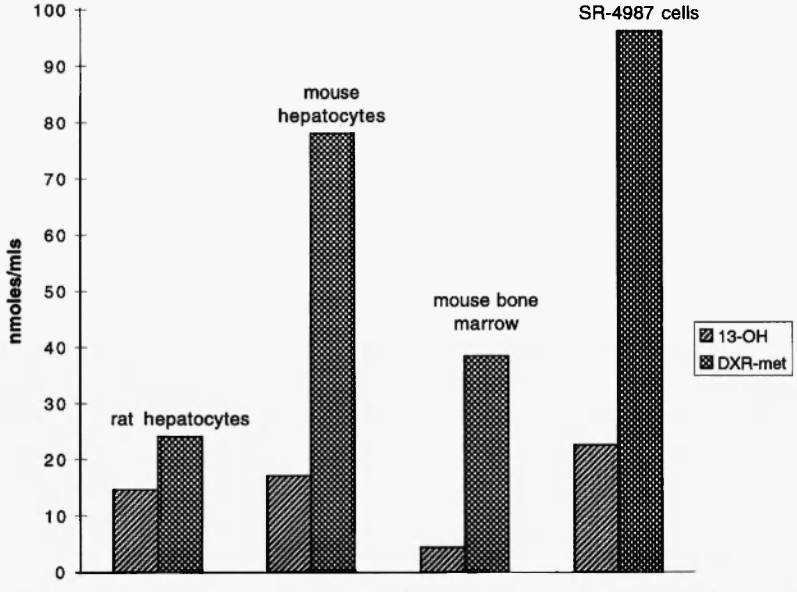

Fig. 3: Production of metabolites in different cell systems after 24 hours of exposure to doxorubicin.

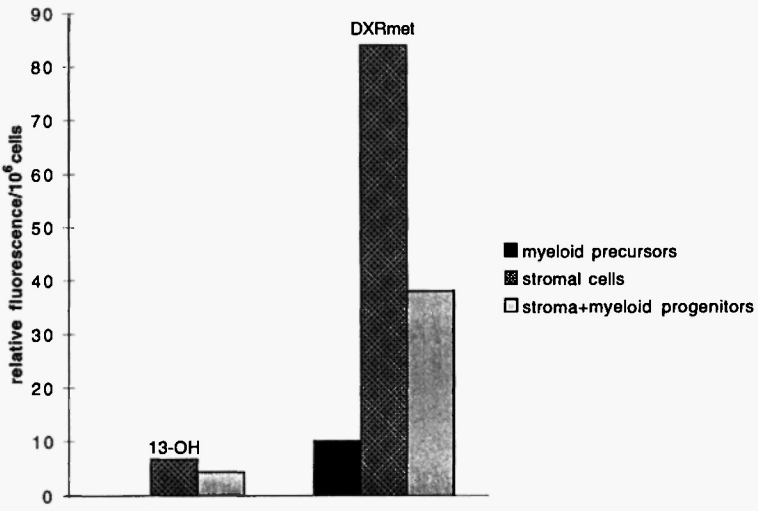

Fig. 4: Production of metabolites in the hemopoietic compartments.

TABLE 1

Production rate of doxorubicin metabolites

	Mouse hepatocytes	SR-4987
Microsomes		
13-OH	0.20	ND
13-OH-one	39	ND
DXR-one	18.3	0.5
13-OH-done	0.70	ND
DXR-met	0.20	1.7
Cytosol		
13-OH	7.2	0.20
13-OH-one	13.7	ND
13-OH-done	ND	0.20

Values are given in pmol/mg protein/min
ND = not detected
13-OH = 13-hydroxydoxorubicin
13-OH-one = 13-hydroxyaglycone
DXR-one = doxorubicin aglycone
13-OH-done = 13-hydroxy-7-deoxyaglycone
DXR-met = unidentified metabolite

DISCUSSION

This study shows that doxorubicin, an anthracycline especially active against hemopoietic malignancies /19/, was metabolized in primary long-term bone marrow cultures by the stromal cell population such as the SR-4987 established stromal cell line. When administered at higher standard dose levels (>100 mg/m^2) peak plasma levels of doxorubicin may reach 6-7 μM /20/, which is a concentration much higher than the dose used in our experiments (0.86 μM). The main metabolites produced were 13-OH and an unidentified metabolite, less toxic than doxorubicin and 13-OH-DXR, that could be the result of the demethylation of the 7-deoxy-aglycone /21/. These results confirm previous studies which showed that bone marrow stroma could play a role in detoxifying xenobiotics /22/. In particular, many studies on benzene toxicity have shown that at low exposure levels, either acutely or chronically, subtle changes in myelopoiesis are involved which appear to depend on local metabolism of the intermediate of benzene initially produced by the liver /23/. Although the biotransformation of xenobiotics occurs mainly in the liver, the bone marrow stromal cells have the capability to metabolize antineoplastic agents such as doxorubicin which act on the hemopoietic compartment as the target tissue. Long-term primary cultures offer the advantage of studying *in vitro* the cellular interactions that occur between hemopoietic and stromal cells *in vivo,* and have been particularly useful for analysis of the role of colony-stimulating factors /24/, characterization of the extracellular matrix and their function in hemopoiesis /25/, and chemical and viral leukemogenesis /26/. This study suggests that they could be a useful model to investigate long-term metabolic activity *in vitro* with respect to chemicals and drugs which are able to concentrate and/or to be active in the immune system.

SR-4987, a murine cell line established from long-term bone marrow culture /27/, has been shown to mimic the stromal population from primary bone marrow cultures in producing the same metabolites in higher quantities, and for this reason it could provide a well-characterized model of stromal cells for studying the metabolism of antineoplastic agents in bone marrow.

Comparison of the metabolites obtained with cytosol and microsomes from murine hepatocytes and SR-4987 stromal cells showed

that 13-OH was produced in the cytosol both from hepatocytes and stromal cells. In contrast, DXR-aglycone was found only in the supernatant from the microsomal fraction.

The aglycone of 7-deoxy-13-OH was produced in microsomes from murine hepatocytes and in cytosol from SR-4987, and a higher rate of production of DXR-met was found in microsomes from stromal cells, suggesting significant O-demethylation activity.

Previous studies indicate that doxorubicin also accumulates in cells of the bone marrow micro-environment, suggesting that the bone marrow stroma may act as a cellular reservoir for high levels of doxorubicin and other anticancer drugs /28,29/. Since these stromal cells may then release the drug into the environment where the more rapidly dividing progenitor cells would be susceptible to antiproliferative drugs /30/, our investigation on the detoxifying activities of stromal cells could be a supportive tool for the evaluation of the real myelotoxicity of such compounds.

The study of the pathways of drug biotransformation in target cell populations standardized in their *in vitro* growth characteristics and culture conditions could be an invaluable support tool in the development of new drugs and new therapeutic approaches.

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