EUROPEAN JOURNAL OF DRUG METABOLISM AND PHARMACOKINETICS, 1986, Vol. 11, No 2, pp. 101-105

Hepatic metabolism of doxorubicin in mice and rats

P. VRIGNAUD, D. LONDOS-GAGLIARDI and J. ROBERT

Fondation Bergonié, Bordeaux, France

Received for publication: May 28, 1985

Key-words: Doxorubicin, mice, rat, hepatic, metabolism

SUMMARY

We have studied the metabolism of doxorubicin in rat and mouse liver, heart and hepatocytes. Doxorubicinol was present in all cases at very low levels and 7-deoxyaglycones were present only in extraphysiological conditions: no aglycones were found either in fresh livers or in hearts of animals treated with the drug, but they were produced in large amounts when the organs were left at room temperature after the death of the animal. Hepatocytes grown in primary cultures or hepatoma cells grown in continuous lines produced no 7-deoxyaglycones. Freshly isolated hepatocytes synthesized small amounts of 7-deoxyaglycones; however, when these hepatocytes were homogenized prior to incubation, high levels of 7-deoxyaglycones were produced. We conclude that 7-deoxyaglycone formation is possible only in injured tissue and is not, therefore, a normal pathway for doxorubicin.

INTRODUCTION

The metabolism of doxorubicin in humans and animals has been much debated since the introduction of this anthracycline antibiotic in clinics. Early studies by the group of Bachur (1-3) evidenced a considerable number of metabolites in urine, plasma, bile or tissues, and in significant amounts. These metabolites were of two types: a reduced glycoside (13-dihydrodoxorubicin, doxorubicinol) and several aglycones or 7-deoxyaglycones originating from the parent drug or its dihydroderivative, and which could be present in a conjugated form. The development of non-degrading techniques of extraction and analysis has dramatically reduced the number of metabolites found in body fluids of humans or laboratory animals. Only doxorubicinol as a unique metabolite has been identified by several authors, especially Israel's group and our group (4-6). At least two steps could be involved in the artefactual production of metabolites of the aglycone type: the extraction, which was performed with acidic mixtures (methanol/0.5 N HCl) by several authors; and thin-layer chromatography, which was frequently performed with acidic solvents (containing as much as 10% acetic acid) on acidic stationary phases (silicagel). It is now generally admitted by the groups working on anthracycline pharmacokinetics that aglycones or 7-deoxyaglycones are barely found in the plasma or urine of patients treated with these drugs.

It has been clearly established, however, that the liver potentially had the capacity of enzymatic cleavage of anthracyclines to aglycones (7, 8). This glycosidase activity has been demonstrated as taking place in a particulate microsomal fraction. When looking for this activity in cultured hepatocytes, we were led to evaluate this metabolic pathway in mice and rats *in vivo* instead of *in vitro*; our results show that the aglycone formation is obtained only after the liver has been injured and is not an actual pathway of anthracycline metabolism in the living animal.

MATERIALS AND METHODS

Animals

Sprague-Dawley rats and Swiss CD1 mice were purchased from Charles River (Rouen, France).

Send reprint requests to: Dr J. Robert, Fondation Bergonié, 180 rue de Saint-Cenis, F-33076 Bordeaux Cedex, France.

Chemicals

Doxorubicin hydrochloride was given by Roger-Bellon Laboratories. Collagenase was purchased from Boehringer-Mannheim. Media and sera for cell culture originated from Seromed. All chemicals and solvents were of analytical grade.

Experiments with whole animals

Doxorubicin hydrochloride was injected in the tail vein of the animals (10 mg/kg). One or two hours later, the animals were killed and the liver and heart quickly removed. A part of the organs was immediately homogenized with a Potter-Elvehjem system maintained at 0°C. The other part of the organs was left at room temperature in a sterile beaker for 1-4 hours and was then homogenized. Anthracyclines and metabolites were extracted from the homogenates as described below.

Experiments with hepatocytes

For the preparation of isolated or cultured hepatocytes, animals were first anesthetized with 50 mg/kg intraperitoneal pentobarbital. The liver was perfused through the portal vein with Ca^{2+} and Mg^{2+} free Hank's medium, then with 150 ml of 0.05% collagenase in Hank's medium as described by Berry and Friend (9) and by Klaunig et al. (10). The liver was removed, dilacerated and the cells were filtered through a nylon gauze. Hepatocytes were freed from contaminating non-parenchymal cells by repeated low speed centrifugations (45 sec at 800 g). The viability of the cells was assessed by their ability in excluding trypan blue. These hepatocytes were either used as freshly isolated cells and incubated directly with doxorubicin, or seeded on Petri dishes in order to obtain primary cultures. In other experiments, cells were homogenized with a Potter prior to incubation with doxorubicin.

For the primary cultures (11), 7×10^6 living cells were plated on 64 cm^2 Petri dishes in Ham F12 medium supplemented with 20% fetal calf serum. The cultures were performed at 37°C in a CO_2 incubator. Four hours after seeding, the viable cells were attached to the Petri dish, and the medium was replaced with fresh medium. Incubations with doxorubicin (0.1-1 µ/ml) were performed either immediately or 20 hours later and lasted 1-4 h. Anaerobic experiments were performed as follows: hepatocytes were seeded in 25 cm^2 flasks (Falcon); 4 hours after seeding, the medium was replaced by 50 ml nitrogen-saturated medium containing 1 µg/ml doxorubicin; therefore, incubation was performed for 1 h without the medium coming into contact with ambiant air.

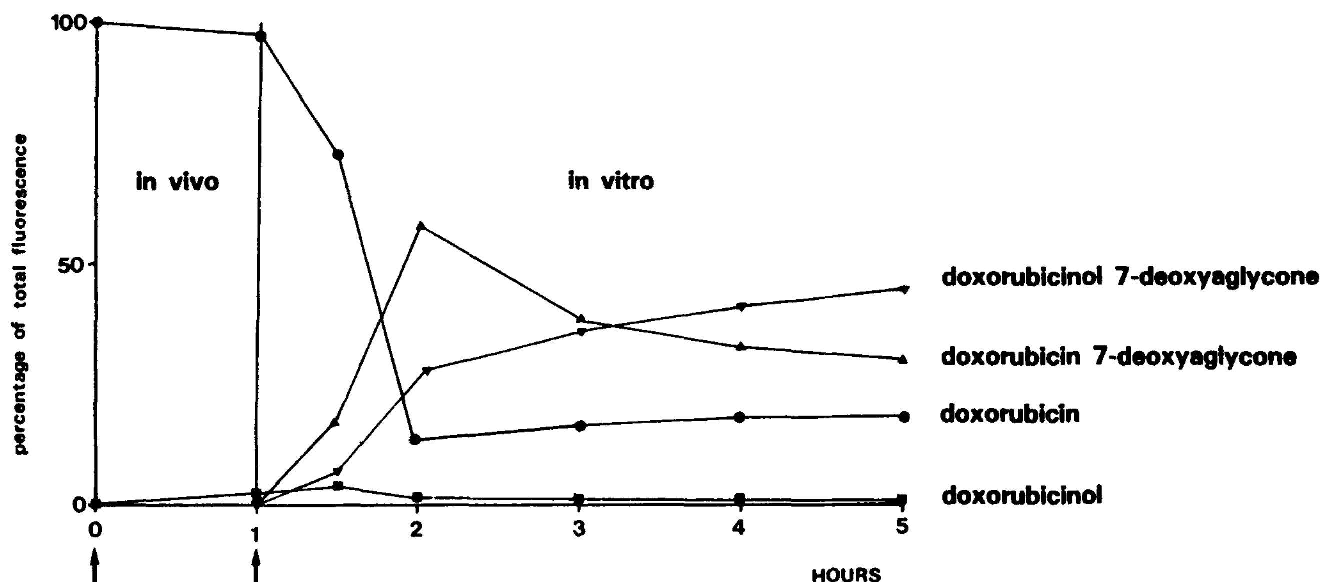

Fig. 1 : Doxorubicin metabolism in pre- and post-mortem rat liver. Doxorubicin (10 mg/kg) was injected at 0 time in the tail vein of rats; 1 hour later, the animal was killed and the liver quickly removed. The liver was immediately homogenized and an aliquot immediately extracted; the remainder of the liver was incubated at 37°C in a sterile flask; aliquots were removed after 30 min., 1 h, 2 h, 3 h and 4 h of incubation. Doxorubicin and metabolites were extracted and analyzed as described in « Materials and Methods ».

Hepatoma cell culture

HTC cells (12) were routinely grown in 64 cm[2] Petri dishes with Dulbecco-modified Eagle's Medium supplemented with 5% fetal calf serum, in a humidified atmosphere containing 5% CO. They were replicated each week and a change of medium occurred once or twice between two passages. Incubation with doxorubicin (0.1-1 µg/ml) was performed for 1 or 4 h.

Extraction and analysis of doxorubicin and its metabolites

Doxorubicin and its metabolites were extracted from homogenates of tissues or cells by the technique of Baurain et al. (13). Homogenates were brought to alkaline pH by addition of 1 vol. of borate buffer, pH 9.8, and immediately extracted with 18 vol. of chloroform/methanol 4/1 (vol/vol). The mixture was shaken and centrifuged ; the lower organic layer was evaporated to dryness under a stream of nitrogen and dissolved in a small amount of the mobile phase of the liquid chromatograph.

High performance liquid chromatography was performed with a Waters apparatus, with a Perkin-Elmer LS1 fluorometer as detector (excitation : 480 mn, emission : 592 mn). The stationary phase was microbondapak-phenyl (Waters) packed in 30 × 0,4 cm metal columns. The mobile phase was a mixture of acetonitrile and 0.1% ammonium formate buffer (32/68, vol/vol) as described by Israel et al. (14).

RESULTS

When the liver or heart of doxorubicin-treated animals was removed, homogenized and extracted immediately after the sacrifice of the animal, the only metabolite which was found was doxorubicinol ; this represented 1-3% of the total anthracycline fluorescence. When the organ was removed, homogenized and left at 37°C in a sterile tube for up to 4 h after sacrifice, the extracts contained large amounts of 7-deoxyaglycones (Fig. 1) : unchanged doxorubicin represented only about 10% of the total fluorescence in the liver one hour after death ; this value then remained stable. Doxorubicin- 7-deoxyaglycone represented more than 50% of total fluorescence one hour after sacrifice, and this percentage decreased thereafter to the benefit of doxorubicinol-7-deoxyaglycone which progressively increased and became the main aglycone 4 hours after death (Fig. 1).

Several experiments were performed with hepatocytes in various conditions. When intact freshly isolated hepatocytes were incubated with doxorubicin for up to 4 h, the main compound in the extracts was doxorubicin itself ; doxorubicin and both 7-deoxyaglycones were also found in the chromatograms, but at very low concentrations (less than 10% of total fluorescence). In contrast, when

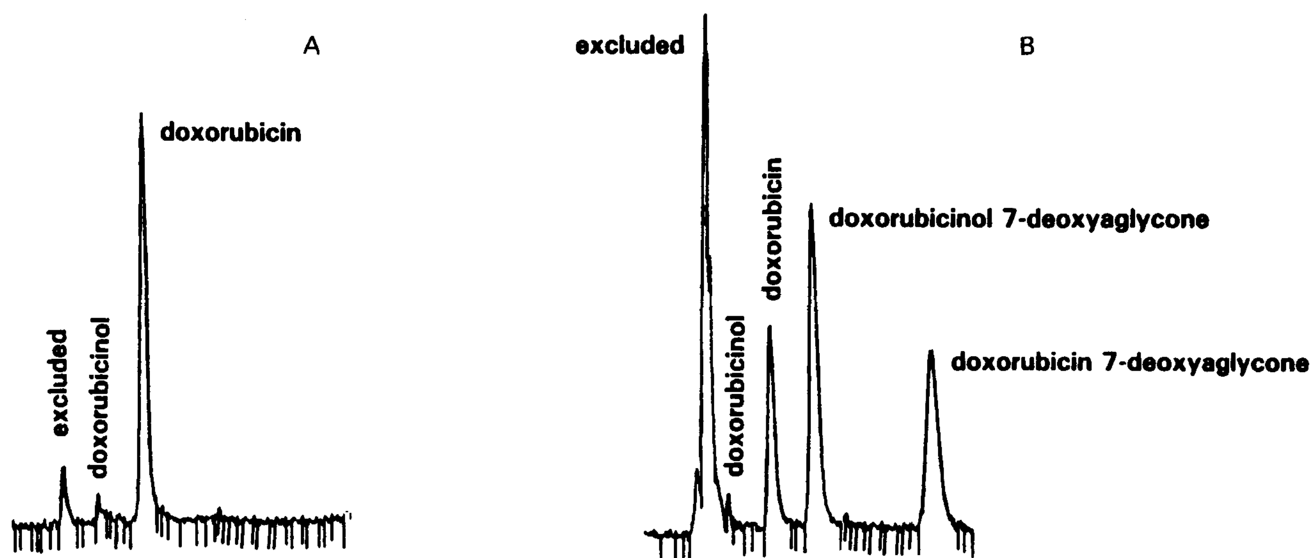

Fig. 2 : Chromatograms of doxorubicin and metabolites.
A. Intact hepatocytes were prepared and cultivated for 24 h as described in « Materials and Methods » and incubated for 1 hour with 1 µg/ml doxorubicin. Doxorubicin and metabolites were then extracted and analyzed.
B. Freshly isolated hepatocytes were homogenized in a Potter and incubated for 1 hour with 1 µg/ml doxorubicin, as described in « Materials and Methods ». Doxorubicin and metabolites were then extracted and analyzed.

the freshly isolated hepatocytes were homogenized prior to incubation with doxorubicin, large amounts of 7-deoxyaglycones were obtained. Fig. 2B shows a typical chromatogram of an extract of homogenized hepatocytes incubated with doxorubicin. When the isolated hepatocytes were plated on Petri dishes, freed 4 hours later from cell debris, and incubated either immediately or 20 h later with doxorubicin, no metabolites were detected either in the cell extracts or in the culture medium; only traces of doxorubicinol (about 1% of total fluorescence) were present (Fig. 2A). When incubation was performed in anaerobic conditions, no change of this metabolic pattern occurred. A similar result was obtained from hepatoma cell cultures incubated with doxorubicin; traces of doxorubicinol were detected as the only metabolite in cell or medium extracts.

DISCUSSION

Israel et al. (15) were the first to suggest that artefactual formation of aglycones in animals and humans fluids or tissues was due to the acidic solvents which are used for extraction and thin-layer chromatography of anthracyclines. As a general feature, it must be emphasized that those authors using thin-layer chromatography (1-3, 16-18) systematically found much more doxorubicin-7-deoxyaglycone than authors using high performance liquid chromatography (4-6, 19-21). The artefactual production of aglycones was evident in a paper by Tavolini and Guarino (16) who found up to 12% of doxorubicin metabolites in the «dosing solution» administered to animals; this never occurs with HPLC techniques.

The presence of aglycones in tissues is less documented than in body fluids. Four authors working with acidic extraction and thin-layer chromatography (16, 22-24) observed high amounts of various aglycones in liver tissue of mice or rats. To our knowledge, no tissue distribution of doxorubicin has been studied with an HPLC technique before the present study, in which no aglycone was observed. We believe that the controversy about liver doxorubicin metabolism might be dissipated by careful *in vivo* and *in vitro* studies. The enzymatic cleavage of anthracyclines to 7-deoxyaglycones by liver microsomes has been demonstrated by the laboratory of Bachur (7, 8). This pathway, however, does not seem functional in normal conditions. This was in fact recognized by Loveless et al. (8) who pointed out that « *in vivo*, relatively little of either adriamycin or daunorubicin is in the aglycone form», and that «the role of these activities in normal cellular physiology is unknown». It has to be emphasized that several types of cultured cells have been studied (25-29), and that they never presented a pathway for the transformation of doxorubicin to 7-deoxyaglycones. We think that this phenomenon may, in fact, represent the general physiological situation, and not a degenerative evolution of the tissue in culture, as has been suggested.

Neither the rat liver *in situ* nor the intact hepatocytes synthesize aglycones; however, homogenization of the liver or of the hepatocytes triggers aglycone synthesis. The most probable hypothesis that can be made is the sequestration of the glycosidases in a cell compartment which is not available for anthracyclines in the intact cell. The ocurrence of small amounts of 7-deoxyaglycones after incubation of freshly isolated hepatocytes may be explained by the fact that not all cells are intact (90% viable cells after trypan blue exclusion). Similarly, Hartman (30) and Gewirtz (31) observed significant amounts of 7-deoxyaglycones after incubation of freshly isolated hepatocytes with doxorubicin. These observations cannot be attributed to technical artefacts since an HPLC technique was used. We think that cytolysis may have occurred between cell isolation and the end of incubation with doxorubicin. Spontaneous, discrete cytolysis may also explain the fact that an isolated perfused rat liver contains signficant amounts of 7-deoxyaglycones after injection of doxorubicin in the system (F. Ballet, personal communication).

The primary culture of hepatocytes in anaerobic conditions did not enhance the formation of 7-deoxyaglycones; this had already been observed in sarcoma cells by Kennedy et al. (32). The absence of biotransformation of doxorubicin that we observed in cultured cells is therefore not due to the oxygen inhibition of the reductive glycosidases (7).

ACKNOWLEDGEMENTS

This work was supported by grants from the Association pour la Recherche sur le Cancer, and from the Ligue Nationale Française contre le Cancer. We are grateful to Ms S. Jean for excellent technical assistance.

REFERENCES

1. Benjamin R.S., Riggs C.E. and Bachur N.R. (1973): Pharmacokinetics and metabolism of adriamycin in man. Clin. Pharmacol. Ther., *14*, 592-600.
2. Takanashi S. and Bachur N.R. (1976): Adriamycin metabolism in man. Evidence from urinary metabolites. Drug Metab. Dispos., *4*, 79-87.
3. Benjamin R.S., Riggs C.E. and Bachur N.R. (1977): Plasma pharmacokinetics of adriamycin and its metabo-

lites in humans with normal hepatic and renal function. Cancer Res., *37*, 1416-1420.

4. Wilkinson P.M., Israel M., Pegg W.J. and Frei E. (1979): Comparative metabolism and excretion of adriamycin in man, monkey and rat. Cancer Chemother Pharmacol., *2*, 121-125.
5. Israel M., Wilkinson P.M., Pegg W.J. and Frei E. (1978): Hepatobiliary metabolism and excretion of adriamycin and N-trifluoroacetyl adriamycin-14-valerate in the rat. Cancer Res., *38*, 365-370.
6. Robert J., Iliadis A., Hoerni B., Cano J.P. and Lagarde C. (1982): Pharmacokinetics of adriamycin in patients with breast cancer: correlation between pharmacokinetic parameters and clinical short-term response. Eur. J. Cancer Clin. Oncol., *18*, 739-745.
7. Bachur N.R. and Gee M. (1976): Microsomal reductive glycosidase. J. Pharmacol. Exp. Ther., *197*, 681-686.
8. Loveless H., Arena E., Felsted R.L. and Bachur N.R. (1978): Comparative mammalian metabolism of adriamycin and daunorubicin. Cancer Res., *38*, 593-598.
9. Berry M.N. and Friend D.S. (1969): High yield preparation of isolated rat liver parenchymal cells. J. Cell Biol., *43*, 506-520.
10. Klaunig J.E., Goldblatt P.J., Hinton D.E., Lipsky M.M. Chako J. and Trump B.F. (1981): Mouse liver cell culture. I. Hepatocyte isolation. In Vitro, *17*, 913-925.
11. Klaunig J.E., Goldblatt P.J., Hinton D.E., Lipsky M.M. and Trump B.P. (1981): Mouse liver cell culture. II. Primary culture. In Vitro, *17*, 926-934.
12. Thompson E.B., Tomkins G.M. and Curran J.S. (1966): Induction of tyrosine-ketoglutarate transaminase by steroid hormones. Proc. Natl. Acad. Sci. USA, *58*, 296-303.
13. Baurain R., Zenebergh A., Trouet A. (1978): Cellular uptake and metabolism of daunorubicin as determined by high pressure liquid chromatography: application to L1210 cells. J. Chromatogr., *157*, 331-336.
14. Israel M., Pegg W.J., Wilkinson P.M. and Garnick M.B. (1978): Liquid chromatographic analysis of adriamycin and metabolites in biological fluids. J. Liquid Chromatogr., *1*, 795-809.
15. Israel M., Pegg W.J., Wilkinson P.M. and Garnick M.B. (1978): HPLC applications in the analysis of adriamycin and analysis in biological fluids. *In:* Biological biomedical applications of liquid chromatography. Hawk J.L., ed. New York: Marcel Dekker, 413-428.
16. Tavoloni N. and Guarino A.M. (1980): Disposition and metabolism of adriamycin in the rat. Pharmacology, *21*, 244-255.
17. Riggs C.E., Benjamin R.S., Serpick A.A. and Bachur N.R. (1977): Biliary disposition of adriamycin. Clin. Pharmacol Ther., *22*, 234-241.
18. Ehninger G., Stocker H.J., Proksch B. and Wilms K. (1980): Die Pharmakokinetik von Adriamycin und Adriamycin-Metaboliten. Klin. Wochenschr., *58*, 927-934.
19. Piazza E., Donelli M.G., Broggoni M., Jessa C., Natale N., Ottolenghi L., Marsoni S., Libretti A., Manzioni C. and Morasca L. (1980): Early-phase pharmacokinetics of doxorubicin in plasma of cancer patients during single or multiple drug therapy. Cancer Treat. Rep., *64*, 845-854.
20. Gil P., Favre R., Durand A., Iliadis A., Cano J.P. and Carcassonne Y. (1983): Time dependency of adriamycin and adriamycinol kinetics. Cancer Chemother Pharmacol., *10*, 120-124.
21. Robert J., Vrignaud P., Iliadis A., Eghbali H. and Hoerni B. (1983): Etude pharmacocinétique de la doxorubicine dans le traitement des lymphomes malins non-Hodgkiniens. Nouv. Rev. Fr. Hématol., *25*, 91-95.
22. Egorin M.J., Clawson R.E., Ross L.A., Chou F.T.E., Andrews P.A. and Bachur N.R. (1981): Disposition and metabolism of adriamycin octanoylhydrazone in mice and rabbits. Drug Metab. Dispos., *9*, 240-245.
23. Bolanowska N. and Gessner T. (1982): Body residue and metabolism of adriamycin and daunorubicin in control and phenobarbital-pretreated mice. Xenobiotica, *12*, 125-136.
24. Arcamone F., Lazzati M., Vicario G.P. and Zini G. (1984): Disposition of ^{14}C-labelled 4'-epidoxorubicin and doxorubicin in the rat. A comparative study. Cancer Chemother Pharmacol., *12*, 157-166.
25. Noel G., Peterson C., Trouet A. and Tulkens P. (1978): Uptake and subcellular localization of daunorubicin and adriamycin in cultured fibroblasts. Eur. J. Cancer, *14*, 363-368.
26. Zenebergh A., Baurain R. and Trouet A. (1982): Cellular pharmacokinetics of aclacinomycin A in cultured L1210 cells. Comparison with daunorubicin and doxorubicin. Cancer Chemother Pharmacol., *8*, 243-249.
27. Egorin M.J., Clawson R.C., Ross L.A. and Bachur N.R. (1980): Cellular Pharmacology of N,N-dimethyldaunorubicin and N,N-dimethyladriamycin. Cancer Res., *40*, 1928-1933.
28. Nguyen-Ngoc T., Vrignaud P. and Robert J. (1984): Cellular pharmacokinetics of doxorubicin in cultured mouse sarcoma cells originating from autochthonous tumors. Oncology, *41*, 55-60.
29. Vrignaud P., Londos-Gagliardi D. and Robert J. (1986): Cellular pharmacology of doxorubicin in sensitive and resistant rat glioblastoma cells in culture. Oncology, *43*, 60-66.
30. Hartman N., Basseches P.J. and Powis G. (1982): Effect of cyclophosphamide pretreatment on the short-term disposition and biliary excretion of adriamycin metabolites in rat. Cancer Chemother Pharmacol., *10*, 11-15.
31. Gewirtz G.A. (1984): Metabolism anthracycline antibiotics in rat hepatocytes. Proc. Am. Ass. Cancer Res., *25*, 293.
32. Kennedy K.A., Siegfried J.M. Sartorelli A.C. and Tritton T.R. (1983): Effects of anthracyclines on oxygenated and hypoxic tumor cells. Cancer Res., *43*, 54-59.